University of Alberta

Human Glioma Immunology and Immunogene Therapy

by

Ian Frederick Parney ©

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partial fulfillment of the requirements for the degree of Doctor of
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Let us, then, be up and doing
With a heart for any fate;
Still achieving, still pursuing
Learn to labor and to wait.

- Henry Wadsworth Longfellow

University of Alberta

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Human Glioma Immunology and Immunogene Therapy" submitted by Ian Frederick Parney in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Experimental Surgery.

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Abstract

Gliomas are the most common primary central nervous system tumors in humans. Despite intensive efforts to improve standard therapy, the prognosis for most glioma patients remains grim. This has led to a search for novel therapeutic strategies reflecting a concomitant explosion in molecular biologic knowledge. In particular, interest in glioma immunotherapy has been rejuvenated by increased insight into molecular immunology. This thesis represents a series of experiments that capitalize on these new data by examining novel aspects of human glioma immunobiology and describing a new method for stimulating anti-glioma immune responses (immunogene therapy).

All experiments were performed with human gliomas (as opposed to animal glioma models) in hopes of increasing our data's clinical relevance. This was facilitated by initial studies in which human glioma cell culture methods were improved. Using cultured human glioma cells, expression of several key immunologic factors was examined. Finally, efforts were made to genetically alter glioma cells' immunogenicity in order to stimulate anti-glioma immunity (immunogene therapy).

Using a simple modification to standard techniques, success rates for establishing human glioma cultures were nearly doubled. Immunobiologic studies with these cultured cells indicated that, while human gliomas are potentially sensitive to anti-tumor immunity, they secrete factors that inhibit immune response activation. Utilizing genetically engineered retroviruses, pro-

inflammatory genes could be efficiently transferred to human glioma cells *in vitro*. Vaccination with these immunogenetically altered glioma cells markedly inhibited growth of pre-established wild type tumors in a novel human lymphocyte / severe combined immunodeficient mouse model.

Based on these studies, it can be concluded that stimulating effective immune responses against human gliomas should be possible. Vaccination with human glioma cells genetically altered to increase their immunogenicity (immunogene therapy) may be one way to accomplish this. The studies in this thesis suggest that human glioma immunogene therapy is both feasible and promising. This has set the stage for pilot glioma immunogene therapy clinical trials. In addition, this work has suggested several other avenues of human glioma immunology investigation that may lead to novel glioma immunotherapy methods.

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List of Abbreviations

AnV	Annexin V
APC	Antigen Presenting Cell
Ca^{++}	Ionized Calcium
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CNS	Central Nervous System
CTL	Cytotoxic T Lymphocyte
DNA	Deoxyribonucleic Acid
DMEM	Dulbecco's Modified Eagle's Media
EMEM	Eagle's Modified Eagle's Media
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Flow Automated Cytometric Stream
FasL	Fas-Ligand
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
HBSS	Hank's Buffered Saline Solution
HSV-tk	Herpes Simplex Virus Thymidine Kinase
hu-PBL	Human Peripheral Blood Lymphocyte
IF-Buffer	Immunofluorescence Buffer
Ig	Immunoglobulin

IL-2	Interleukin-2
IL-2R	Interleukin-2 Receptor
IL-3	Interleukin-3
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-12	Interleukin-12
IFN-α	Interferon Alpha
IFN-β	Interferon Beta
IFN-γ	Interferon Gamma
IGF-1	Insulin-Like Growth Factor 1
IGF-1R	Insulin-Like Growth Factor 1 Receptor
IRES	Internal Ribosome Entry Site
IUdR	Iododeoxyuridine
GFAP	Glial Fibrillary Acidic Protein
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GSF	Glioma-Derived Suppressor Factor
LAK	Lymphokine-Activated Killer Cell
LTR	Long Terminal Repeat
MHC	Major Histocompatibility Complex
MLV	Maloney Leukemia Virus
mRNA	Messenger Ribonucleic Acid

NGF	Nerve Growth Factor
NGFR	Nerve Growth Factor Receptor
NK Cells	Natural Killer Cells
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCV	Procarbazine, CCNU, Vincristine
PDGF	Platelet-Derived Growth Factor
PGE2	Prostaglandin E_2
PHA	Phytohemoagglutinin
PI	Propidium Iodide
rtPCR	Reverse Transcriptase Polymerase Chain Reaction
SCID	Severe Combined Immunodeficiency
SV40	Simian Virus 40
T_H	Helper T Cells
T_K	Killer T Cells
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor Beta
TIL	Tumor-Infiltrating Lymphocyte
TNF-α	Tumor Necrosis Factor Alpha
TNFR	Tumor Necrosis Factor Receptor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
VEGF	Vascular Endothelial Growth Factor

Chapter 1

Glioma Immunology and Immunotherapy: A Review

Introduction

Gliomas are tumors arising from glial cells (particularly astrocytes and oligodendrocytes). They are the most common primary central nervous system neoplasm, with an incidence between 2.1 to 7.1 per 100 000 per year (19). In many industrialized nations, increasing incidence has been reported, particularly among the elderly (39, 67, 135). However, this may reflect an increased diagnosis through improved imaging technology rather than a true increase in incidence (168).

Gliomas carry a dismal prognosis. Mean survival following diagnosis of a glioblastoma multiforme tumor is less then one year with the best treatment currently available (32, 50). This has not changed significantly in three decades. Median survival in anaplastic astrocytoma patients is only marginally better, with best median survival reported in the literature being three years (38, 120, 226). Even patients harboring relatively chemosensitive oligdodendrogliomas (30, 128), largely succumb to their tumors eventually and ten-year survival is poor. As can be expected, patients with recurrent gliomas face even worse prognoses. For example, a recent phase II study examining prolonged oral etoposide (VP16) treatment in patients with recurrent gliomas demonstrated mean survivals of 11.6, 35.9, and 51.9 weeks, respectively, for recurrent glioblastoma multiforme, anaplastic astrocytoma, and anaplastic oligodendroglioma patients (55).

To try and improve this dismal prognosis, many variations on standard therapeutic modalities (surgery, radiation, and chemotherapy) have been investigated. Although surgery is a mainstay of glioma treatment, the role of aggressive surgical resection in gliomas remains controversial (153). Most authors feel that maximal cytoreduction is beneficial (157, 248) and increases the effectiveness of adjunctive chemo- or radiation therapy (119, 191). However, benefits afforded by aggressive resection may be lost in elderly and debilitated patients (99). In the future, advances in image guided surgery and intra-operative electrophysiologic mapping may improve the ability to safely effect gross total resections for gliomas (199, 220).

Post-operative whole brain radiotherapy is standard for most glioma patients and does prolong survival (32). Most attempts to improve outcome further with hyperfractionationated and accelerated radiotherapy have thus far been ineffective or toxic (62, 94), although one phase II trial did show improved outcome in anaplastic astrocytomas (212). Similarly, radiosensitizers such as iododeoxyuridine (IUdR) have been moderately effective in prolonging disease free survival in anaplastic astrocytomas (226) but not glioblastomas (225). A recent randomized controlled trial demonstrated limited benefit for brachytherapy in glioma treatment (12). However, stereotactic radiosurgery (another form of high dose local radiotherapy) may be more promising (124, 206).

Chemotherapy with nitrosoureas (BCNU, CCNU) has a moderately beneficial impact on survival in patients with glioblastoma multiforme tumors and is often utilized as adjunctive therapy. It has been recently recognized that oligodendrogliomas are relatively chemosensitive to PCV (procarbazine, CCNU, and vincristine) (30, 31, 128). In a phase II trial, mean survival for responders (75% of patients treated) was greater than four years (31). Because the blood brain barrier excludes many chemotherapeutic agents, several alternate approaches to drug delivery for CNS chemotherapy have been investigated. For example, interstitial chemotherapy with drug-impregnated biodegradable polymer wafers shows considerable promise for many gliomas (22, 23). In contrast, intra-arterial delivery of chemotherapeutic agents has produced conflicting results in several studies. Some show unacceptably high toxicities (65, 131) while others show improved outcomes (154, 171).

Unfortunately, improvements in standard therapy have not significantly altered prognosis for most glioma patients. However, recent years have seen an exponential explosion in knowledge about glioma genetics and biology that may lead to more effective therapeutic strategies. Cytogenetic abnormalities such as hyperploidy, gain of chromosome 7, loss of chromosomes 10, 13, 17, and 22, and gain of double minutes are frequently observed (92, 244). Mutation or over expression of oncogenes such as platelet-derived growth factor (PDGF, c-cis) (242) and epidermal growth factor receptor (EGFR, v-erb) (35, 117) leading to increased Ras pathway activation and subsequent cell proliferation (72) are common. Similarly, loss or mutation of tumor suppressor genes such as p53, p16, and retinoblastoma protein have been described in astrocytomas (96, 117). These losses, along with up-regulation of cyclin D and/or amplification of CDK4, can lead to dysregulation of cell cycle control (40, 74). These abnormalities are further compounded in glioblastomas by frequent tumor suppressor gene loss on chromosome 10, including the PTEN/MMAC1 gene (121, 210).

There appears to be a hierarchy of molecular genetic abnormalities that corresponds with glioma grade (127). p53 loss occurs early (in grade II astrocytomas) while p16 loss, retinoblastoma protein loss, and cyclin D up-regulation are associated with the transition to anaplastic (grade III) astrocytomas. Finally, EGFR amplification and tumor suppressor gene(s) loss on chromosome 10 are strongly associated with glioblastomas (117, 137, 229).

In addition to molecular genetic abnormalities in gliomas, new insight has been gained into glioma growth and spread. Angiogenic factors expressed by gliomas, including vascular endothelial growth factor (VEGF), promote new blood vessel growth to supply tumors (165, 241). Changes in cell adhesion molecules along with upregulation of matrix metalloproteinases allow for increased motility and invasion within the CNS (57).

New insights into glioma molecular biology have suggested many novel treatment strategies (102, 260, 262). One experimental strategy rejuvenated by this explosion of knowledge is glioma immunotherapy. Stimulating the immune system to combat cancer has long been attractive as it has the potential to be a "magic bullet" that kills tumor cells while leaving normal cells unharmed. Unfortunately, this goal has remained elusive. However, recent advances in immunology, oncology, and molecular biology make this once impossible task now seem possible. Because of their dismal prognosis with standard therapy, this approach is particularly attractive for gliomas. In the remainder of this chapter, we will review basic immunology, CNS immunology, glioma immunobiology, and glioma immunotherapy.

Basic Immunology

Immunity can be defined as the ability to resist a dangerous infection or growth through elimination of the dangerous substance or cell. The immune system is a diffuse body system concerned with protection from infectious invaders and internal threats such as neoplasia. Anatomically, it consists of primary lymphoid organs (bone marrow, thymus) involved with generation and preliminary maturation of immune cells and secondary lymphoid organs (lymph nodes, spleen) involved with terminal differentiation and activation of immune cells. Lymphocytes are the key immune cells. Different lymphocyte subsets have been described, including T cells, B cells, and NK (natural killer) cells. There is a constant lymphocyte flux between the periphery and secondary lymphoid organs via both blood and lymph (29).

Active immune reactions are concerned with eliminating complex biologic molecules (antigens) that are recognized as "not self." This is mediated by binding to a number of highly polymorphic lymphocyte proteins (T and B cell receptors). There are two main types of active immune responses: humoral and cellular. B cells secreting antibodies that bind to specific foreign antigens mediate humoral responses. T cells binding directly to foreign antigens on target cells mediate cellular responses. Active immune responses involve four distinct phases: activation, proliferation, effector, and memory.

The first phase of immune responses (lymphocyte activation) involves complex interactions between several cell types and regulatory proteins. At least two signals are required. In humoral responses, the binding of a foreign antigen to a receptor (an immunoglobulin) on a B cell surface provides the first signal. In addition, a second signal must be provided by a nearby helper T cell (T_H). Similarly, cell-mediated responses require two signals. The binding of a foreign antigen to the T cell receptor on a killer T cell (T_K) provides the first signal while a nearby T_H cell provides the second signal.

T cell activation itself is a complex process involving several signals. As indicated above, different T cell subsets subserve different immune functions. Generally speaking, T_H cells ($CD4^+$) provide secondary signals required to activate both humoral and cellular immune responses. T_K cells ($CD8^+$) mediate active cytotoxic immunity (159). The first signal required for both T_H and T_K cell activation is foreign antigen binding to T cell receptors (TCR). Unlike B cells, however, T cells do not recognize "naked" antigen. In order to bind TCR, antigen must be processed and presented in conjunction with a major histocompatibility molecule (MHC). T_K cells recognize antigen in the context of class I MHC while T_H cells recognize antigen with class II MHC. In the past ten years, it has been appreciated that antigen presentation alone is not adequate to activate T cells. In addition, a second "costimulatory" signal is required (183). Antigen-primed T cells that do not receive costimulatory signals enter a state of unresponsiveness called "anergy" (58). The most important costimulatory molecules appear to be B7-1 (CD80) and B7-2 (CD86) which bind to a receptor (CD28) on the T cell surface (6, 53, 203, 250).

In addition to T cells and B cells, antigen-presenting cells (APC's) are also critical to activating immune reactions. As their name implies, APC's present antigen to lymphocytes (B and T cells) and are important immune response mediators. They can be functionally divided into "professional" and "non-professional" APC's. Virtually all cells in the body have some potential to present antigen and can be considered non-professional APC's. Professional APC's are a subset of myeloid-lineage cells (including monocyte / macrophages, dendritic cells, microglia, and neutrophils) that have special characteristics that make them highly efficient immune response stimulators (155, 164, 176). Their highly phagocytic nature provides them with a constant antigen supply and they are very efficient at processing these antigens for presentation. Like most nucleated cells, they express class I MHC. However, unlike most cells, they also express high levels of class II MHC. This makes them uniquely able to stimulate T_H cells. In addition, they are a rich source of T cell costimulatory molecules such as B7-1 and B7-2 and they secrete many cytokines that can regulate immune reactions. Underlying their importance in immune responses, it has recently been proposed that a professional APC-derived third signal (the "danger signal") may be necessary for T cell activation in addition to antigen presentation and costimulation (54, 138, 139).

Finally, in addition to the cellular interactions outlined above, many cytokines play important roles in immune responses. Cytokines are small proteins with immunoregulatory functions. Some, such as interleukin-2 (IL-2), are critical for T cell proliferation (234). Others, such as transforming growth factor beta (TGF-β), are immunosuppressive and inhibit lymphocyte proliferation (51). Lymphocytes (particularly T_H cells) can be categorized by their individual cytokine profiles (149). For example, T_H1 cells secrete cytokines such as interferon-gamma (IFN-γ) and interleukin-12 (IL-12) and are associated with cell-mediated immunity. T_H2 cells secrete interleukin-4 (IL-4), interleukin-6 (IL-6), and interleukin-10 (IL-10) and are associated with humoral immune responses.

Once T cells or B cells have been appropriately activated, proliferation occurs that leads to an expanded population of mature effector lymphocytes capable of eliminating foreign antigen. In the case of humoral responses, antibodies secreted by effector B cells bind foreign antigens which leads to increased phagocytosis and elimination by cells such as macrophages, neutrophils, and even B cells themselves. Humoral responses are particularly effective at eliminating highly foreign antigens such as those associated with bacterial infections. In contrast, mature cytotoxic effector cells (largely T cells) mediate cellular immunity by inducing programmed cell death (apoptosis) in target cells expressing specific foreign antigens. This is accomplished by one of two pathways: Fas-ligand released by cytotoxic T cells may induce apoptosis by binding Fas on target cells or perforins released by cytotoxic T cells perforate target cells and allow T cell-derived granzymes to enter and induce apoptosis (5, 95). Cellular responses are particular effective at eliminating "less foreign" antigens (those with more similarity to "self") such as those present on tumor cells.

Memory is the final phase of active immune responses. When the foreign antigen that stimulated the immune response has been cleared, most of the effector lymphocytes are eliminated. However, a small population persists and evolves into "memory" lymphocytes. These population remains behind so that it can rapidly respond to subsequent challenge with the same antigen (1). Memory is the principle behind the immunity seen after vaccination or exposure to many infectious diseases.

In summary, immunity involves recognizing and eliminating dangerous antigens (infectious or neoplastic). It involves a diffuse body system consisting of primary and secondary immune organs and is mediated largely by lymphocytes. Immune responses can be thought of as occurring in four phases: activation, proliferation, effector, and memory. Appropriate antigen presentation activates lymphocytes that recognize specific antigens and causes them to proliferate and become active effector cells. This involves several intra- and intercellular signals. Mature effector lymphocytes eliminate foreign antigens (and/or cells bearing foreign antigens) through either humoral or cellular immune responses. After foreign antigen has been eliminated, a subpopulation of memory lymphocytes remains that can quickly respond to subsequent challenge with the same antigen. The four phases of immune responses are shown schematically in **Figure 1-1**.

Central Nervous System Immunology

The central nervous system has classically been considered an immunologically privileged site. This is based on several observations. First, in his seminal studies on transplantation immunology, Dr. Peter Medawar demonstrated that allogeneic tissue grafts were not rejected when placed within the brains of experimental animals (141). Several explanations for this phenomenon were subsequently proposed. It was felt that the blood brain barrier probably prevented lymphocyte entry, thus limiting immune responses within the CNS. Further evidence for isolation from the immune system was found in the fact that brain and spinal cord did not appear to have any draining lymphatics appeared to confirm the central nervous system's isolation from the immune system (13). Finally, despite Cajal's identification of microglia as macrophage-like cells within the brain in 1913, it was felt that the CNS did not possess resident antigen-presenting cells (215).

Despite the evidence outlined above, many recent studies have questioned the degree of immune privilege within the CNS. To a large degree, the proceeding arguments in favor of CNS immune privilege do not stand up to closer scrutiny. First, many studies have demonstrated intracerebral xeno- and allograft rejection (66, 200, 201) unless it is performed in immunoincompetent (27, 170) or immunosuppressed (108, 196) animals. It has now been recognized that many pathologic (viral infection, neoplasia, multiple sclerosis) and experimental

(experimental allergic encephalitis) conditions involve blood brain barrier breakdown and free lymphocyte traffic into the CNS as a result (28). It has also become apparent that continuous ~~and~~ highly regulated connections do exist between cerebrospinal fluid compartments and cervical lymphatics (37, 251). Finally, the role of microglia as ubiquitous professional antigen presenting cells within the central nervous system has been confirmed (47, 56, 73, 215). In addition to these findings, other evidence reinforces the functional and teleologic connections between the central nervous system and the immune system. These include pathways for neuroendocrine-immune interactions (174) and molecular similarities between CNS proteins such as myelin/oligodendrocyte glycoprotein and the immunoglobulin gene superfamily (211).

Nevertheless, the central nervous system has a complex microenvironment in which immune responses are modulated. In general, cell mediated immune responses are diminished while humoral responses are normal or increased (37). This may in part be due to relatively low expression of MHC molecules on most neurons and glia except microglia (113, 114). Modulation by cytokines may also occur, although this appears to be more prominent in central nervous system tumors (231). However, while some types of immunity may be down regulated, it is clear that immune responses can and do occur within the CNS.

Glioma Immunobiology

Confirming that immune reactions can occur within the central nervous system has important implications for CNS oncology. In particular, it suggests that it may be possible to generate effective immune responses against gliomas. This concept has been recently bolstered by studies identifying tumor-associated antigens expressed by gliomas such as tenascin, GP240, altered EGFR isoforms (EGFRvIII), and several previously identified melanoma-associated antigens (tyrosinase, tyrosinase-related proteins 1 and 2, gp100, MAGE-1, MAGE-3) (33, 110). These antigens provide a potential stimulus for anti-glioma immunity.

Although gliomas express tumor-associated antigens, their ability to present these antigens to T cells is controversial. This relates to their MHC expression (or lack thereof). Some authors have reported that human gliomas express class I MHC (145, 190). However, in a series of well controlled studies, Lampson *et al.*. demonstrated very low or unmeasurable class I MHC expression by human gliomas *in vitro* and *in situ* using immunohistochemistry (112, 114, 240). It may be possible to explain these divergent results by differences in staining techniques, controls, and/or *in vitro* / *in vivo* conditions (112). However, other evidence suggests that at least some gliomas have some degree of class I MHC expression. For example, it is well recognized that some tumors down regulate class I MHC expression to avoid immune surveillance (178). Such tumors usually completely lose class I MHC expression through mutation or deletion of β_2 microglobulin (48, 179). Cell lines derived from these tumors are unable to express class I MHC, even when exposed to appropriate stimuli such as IFN-γ. In contrast, Lampson *et al.* have demonstrated that gliomas increase class I MHC expression both *in vitro* and *in vivo* following IFN-γ exposure (113). This suggests that gliomas have not completely lost class I MHC expression. Rather, they may have low background levels of class I MHC expression that can be upregulated when they are exposed to appropriate stimuli. This background expression level may be too low for reliable detection by immunohistochemistry. However, other more sensitive techniques may be able to detect it. In our own laboratory, we have recently demonstrated class I MHC expression in 16/16 early passage human glioma cultures using highly sensitive flow cytometry (162). Taken together, it seems likely that human gliomas do express low levels of class I MHC that can be upregulated under appropriate conditions. Therefore, gliomas are likely able to present antigens to T_K cells via class I MHC.

Unlike T_K cells, T_H cells require class II MHC expression for antigen presentation. Class II MHC molecules are generally provided by professional antigen presenting cells (155). Although both normal and neoplastic astrocytes are able to express low levels of class II MHC when stimulated by IFN-γ (81, 173), it appears that microglia are the usual class II MHC^+ cells in the central nervous system (56, 215). Interestingly, microglia / macrophages are present in increased numbers in both rodent glioma models (148) and in human gliomas *in situ* (118, 184, 186). The absence of anti-glioma immunity suggests that these microglia are not functioning as effective immunostimulating cells. Indeed, it is possible that they may be subverted by the tumor into secreting factors that support glioma growth (134). Nevertheless, they are a readily available source of class I / II MHC + antigen presenting cells for gliomas.

Antigen presentation and T cell activation alone are not sufficient for successful cell-mediated immune responses. Cell killing by cytotoxic T lymphocytes is also necessary. The primary pathways for cytotoxic T cell-mediated killing are the Fas/Fas-ligand pathway and the perforin/granzyme pathway (5, 95). Of these, the Fas/Fas-ligand pathway is felt to be more important physiologically as, unlike the perforin/granzyme pathway, it does not depend on supra-physiological Ca^{++} levels (189). If glioma cells are to be killed by cytotoxic T cells, they must express Fas. Most human gliomas express Fas *in vitro* and *in vivo* (3, 237-239). Fas^{+} human glioma cell lines exposed to anti-Fas antibodies or Fas-ligand undergo apoptosis (188, 237). Furthermore, lymphocyte cytocidal activity *vs.* an allogeneic human glioma cell line has been shown to be mediated by Fas / Fas-ligand interactions (216). These reports suggest that human gliomas are potentially sensitive to Fas / Fas-L-mediated apoptosis and, therefore, the major mechanism for T cell-mediated cytotoxicity.

However, the situation may be more complex. Some authors report Fas-ligand expression in gliomas (64, 188). This is difficult to reconcile the apparent Fas^{+}, Fas-L-sensitive status of most gliomas. In Fas^{+} cells, Fas-L expression suggests insensitivity to Fas-mediated apoptosis. Otherwise, Fas-L expression would be a form of cellular suicide. This insensitivity could significantly impair cytotoxic T cells' ability to kill glioma cells. In addition, Fas-L expression could further suppress immunity by inducing apoptosis in Fas$^+$ naïve T cells (68, 235). Gratas *et al.* have suggested that different subpopulations within single tumors have different Fas / Fas-L expression patterns (64). This may explain the apparent paradox of gliomas expressing both Fas and Fas-L. It is likely, then, that a least a significant proportion of glioma cells are Fas$^+$ / Fas-L$^-$ and, therefore, potentially sensitive to Fas-mediated cytotoxic T cell killing.

Tumor specific antigens, pathways for class I and II MHC-restricted antigen presentation, and Fas expression all suggest that gliomas ought to be sensitive to cell-mediated immune responses. Unfortunately, it is clear that effective anti-tumor cell-mediated immune responses are not generated in glioma patients. Several factors contribute to this. Based on the classic contributions of Brooks, Mahaley, Young and others, it has long been recognized that patients harboring malignant gliomas exhibit depressed cellular immune responses compared to healthy individuals (26, 130, 256). This is reflected in decreased lymphocyte counts, decreased skin-test antigen response, and impaired lymphocyte response to *in vitro* stimulation. Despite this, gliomas are infiltrated by lymphocytes *in situ* to varying degrees (160, 232, 247). This was initially taken as evidence for anti-tumor immunity (180) but subsequent studies could not find a convincing correlation with prognosis (24). More recent evidence suggests that glioma-infiltrating lymphocytes are inactivated (15). Several cellular observations correlate with systemic and intra-tumoral immunosuppression in glioma patients. Lymphocytes from glioma patients exhibit defects in IL-2 and interleukin-2 receptor (IL-2R) expression after stimulation *in vitro* (187). Exogenous IL-2 is not enough to overcome these defects (43), possibly due to additional defects in T cell receptor (TCR) signaling (147).

It seems likely that impaired lymphocyte responses in glioma patients result from secreted immunoinhibitory factors rather than intrinsic lymphocyte defects (140). Glioblastomas secrete several cytokines that could be responsible for these effects (231). In particular, glioblastoma-derived transforming growth factor-β2 (TGF-β2) is known to suppress lymphocyte proliferation (17, 109, 207) and to deactivate macrophages (223). Glioblastomas also produce prostaglandin-E_2 (PGE2) which inhibits T cell responses and suppresses pro-inflammatory cytokine synthesis (52, 198). More recently, IL-10 mRNA has been isolated from human gliomas (83, 156). Interestingly, *in situ* hybridization studies indicate that a large proportion of glioma-derived IL-10 mRNA localizes to glioma-infiltrating microglia (as opposed to glioma cells themselves) (82). Regardless of its cellular source, local IL-10 production (a T_H2 cytokine) could shift immunity to humoral responses, which may be less effective than cell mediated responses at clearing solid tumors such as gliomas. Similar shifts to T_H2 pathways may also be induced by an as yet uncharacterized glioma-derived suppressor factor (GSF) (25, 147, 187).

Glioma immunobiology is complicated by the fact that gliomas appear to express several "proinflammatory" cytokines including IL-6 and interleukin-8 (IL-8) (98, 213, 228, 230). Gliomas do not express IL-6 or IL-8 receptors (231) and their function in gliomas is not immediately clear. IL-6 is a T_H2 cytokine that can stimulate monocyte/macrophage-like cells such as microglia (149, 261). One can speculate that IL-6 secretion may stimulate microglial infiltration into gliomas. Prominent microglial infiltrates have been increasingly recognized in gliomas (118, 184, 186). Similarly, IL-8 is a well known chemotactic factor for inflammatory cells (227). It is possible that IL-6 and IL-8 attract and stimulate specific inflammatory cells that express factors that promote glioma growth. Considerable evidence in the literature supports such interactions between glioma and inflammatory cells. For instance, lymphokine-activated peripheral blood mononuclear cells (PBMC) from glioma patients stimulate glioma proliferation *in vitro* (87). Similarly, IL-6 can induce microglia to secrete IL-10 *in vitro* (204). IL-10 mRNA has been localized to glioma-infiltrating microglia *in situ* (82, 204). IL-10, in turn, can induce glioma proliferation and migration (82). Gliomas' ability to subvert inflammatory cells into pro-glioma functions may explain why patients with increased inflammatory cell infiltrates appear to have a worse prognosis (15).

Thus, a complicated picture of glioma immunology has emerged. Gliomas express tumor-specific antigens and (either by themselves or through interactions with neighboring microglia) have access to pathways for both class I and class II MHC-restricted antigen presentation. Furthermore, glioma cells express Fas and most appear sensitive to Fas-mediated apoptosis (the major pathway for cytotoxic T cell-mediated killing). These factors suggest that gliomas are potentially sensitive to cell-mediated immune responses. However, gliomas express several immunosuppressive factors (TGF-β2, PGE2, and possibly Fas-L) that hinder lymphocyte activation. T_H2 cytokines (IL-6, IL-10) that may shift immunity to less effective humoral responses are also found in gliomas. Finally, complex interactions between gliomas and inflammatory cells, possibly mediated by glioma-derived cytokines such as IL-6 and IL-8, appear to actually promote glioma growth. Glioma immunobiology is summarized schematically in **Figure 1-2**.

Glioma Immunotherapy

The immune system's vast diversity gives it the potential to specifically eradicate abnormal or infected cells while leaving normal tissues unharmed. As a result, immunotherapy has long held out the promise of a "magic bullet" for cancer therapy. Many cancer immunotherapy approaches have been proposed, including administering non-specific immune adjuvants (cytokines), anti-tumor antibodies (serotherapy), anti-tumor lymphocytes (adoptive immunotherapy), and anti-tumor vaccines (active immunotherapy). These are summarized in **Table 1-1**. Variations on all of these strategies have been investigated in glioma patients.

Non-specific immune adjuvants (cytokines) have been studied for many different tumors. The rationale for their administration is twofold (49). First, it is hypothesized that subtherapeutic anti-tumor immune reactions are normally present in cancer patients. It is hoped that systemic cytokine therapy will boost these responses to clinically effective levels. Second, many cytokines have direct anti-tumor activity. Therefore, systemic cytokine administration may inhibit tumor growth independently from immunologic responses. Pre-clinical studies have suggested roles for several cytokines in glioma therapy, including IL-2, IL-4, interferon-α (IFN-α), and tumor necrosis factor-α (TNF-α) (84, 101, 115, 142). Several of theses have undergone clinical trials. IL-2 has largely been evaluated in conjunction with lymphokine activated killer (LAK) cell therapy (see below). Unfortunately, IL-2 therapeutic activity appears to require high doses that result in treatment-limiting cerebral edema (142, 185). In small uncontrolled studies, TNF-α demonstrated modest anti-glioma effects in a subset of patients (136, 254) but conclusive evidence of efficacy has been lacking. Little benefit has been seen for IFN-α administration alone (18, 133). A more recent study combining IFN-α with chemotherapy reported a few long term (5 year) survivors, but treatment limiting neurotoxicity was noted (93). Similar lack luster results have been seen with interferon-β (IFN-β) (233, 259) and IFN-γ (45, 167). Thus, despite promising pre-clinical studies, cytokine administration has produced limited clinical benefit in glioma patients to date.

Serotherapy, another form of immunotherapy, involves administering anti-tumor antibodies to patients. Glioma serotherapy has been extensively investigated and largely reflects the contributions of Dr. Darell Bigner and his colleagues at Duke University. Preclinical studies have identified monoclonal antibodies directed against several glioma-specific antigens (8, 20, 21, 34, 111, 116, 150, 246). Based on these, a number of anti-glioma serotherapy clinical trials have been reported. ^{131}I-labeled murine monoclonal antibodies directed against tenascin (a relatively glioma-specific extracellular matrix protein) have shown promise in phase I/II clinical trials. Response rates up to 51% and mean survivals of 15 - 23 months have been reported with minimal toxicity (14, 182, 194). Preliminary results with ^{131}I-labeled Mel-14 F(ab')$_2$ specific for Gp240 (a chondroitan sulfate-associated antigen expressed on gliomas and melanomas) have shown similar results (14). Definitive assessment will require prospective randomized, controlled trials, but these two serotherapies show significant promise. In addition, monoclonal antibodies directed EGFR mutants expressed by many malignant gliomas (eg. EGFRvIII) have shown promise in pre-clinical studies (125, 175) and await clinical evaluation.

Adoptive immunotherapy involves intravenous or intratumoral infusion of autologous *ex vivo*-stimulated lymphocytes (adoptive transfer). Early trials simply infusing unstimulated autologous lymphocytes intratumorally in glioma patients did not prolong survival (255). Subsequently, efforts were made to improve results by administering LAK cells - lymphocytes stimulated *in vitro* with IL-2 (71). LAK cells can kill glioma cells *in vitro* (85, 86, 106, 152) and prolong survival of rats harboring intracranial gliomas when infused *in vivo* (224). Unfortunately, LAK clinical trials have not demonstrated clear benefits and significant side effects such as cerebral edema due to concomitant high dose IL-2 administration have been frequent (7, 123, 142, 195).

The LAK therapy's failure led to efforts to refine adoptive transfer therapies. One approach involved isolating tumor infiltrating lymphocytes (TIL's), stimulating them *in vitro* with IL-2, then re-administering them to patients. This approach was similar to LAK therapy, but it was hoped that TIL's would be more tumor specific as their origin suggests priming to tumor antigens *in situ*. Although this approach showed promise *in vitro* (70, 143), it has been ineffective in treating intracranial tumors in animal models (197). Because of this and the technical difficulty involved in isolating TIL's, it has not been investigated extensively.

Stimulating lymphocytes with autologous tumor cells to generate class I MHC-restricted cytotoxic T cells (CTL's) is another relatively simple approach to improve adoptive transfer therapies. This has produced effective anti-glioma CTL's *in vitro* (146). More recently, Holladay and colleagues demonstrated improved survival in rats harboring intracerebral RT-2 gliomas after infusing autologous lymphocytes stimulated first *in vivo* by vaccination with irradiated RT-2 cells and then stimulated *in vitro* by RT-2 cell co-culture (77, 78, 80). In a novel twist, Kruse and colleagues have shown that rat 9L gliomas can be eradicated after intra-tumoral infusion of allogeneic lymphocytes stimulated *in vitro* with either 9L cells (172) or simply with 9L-syngeneic MHC molecules (104, 105, 107). Both groups have recently reported small pilot clinical trials that established the feasibility and minimal toxicity their approaches (79, 103). Definitive evaluation of clinical efficacy will require further study.

The final class of immunotherapy that has been studied in glioma treatment is active specific immunotherapy (vaccination). This form of therapy, generally consisting of autologous tumor cell vaccines, was evaluated in several studies in the 1970's and early 1980's but fell out of favor due to lack of efficacy. Of these studies, Trouillas reported the most promising results (221). In a randomized control trial, 65 patients with high-grade astrocytomas were assigned to receive radiation, immunotherapy (autologous tumor cell vaccines with Freund's adjuvant), radiation and immunotherapy, or supportive care. Twenty-four of 28 patients receiving immunotherapy developed delayed hypersensitivity reactions to autologous tumor cells. Furthermore, survival was prolonged for patients receiving radiation and immunotherapy (10.1 months) compared to radiation alone (7.5). However, other studies were less encouraging. Bloom *et al.* reported no increased survival compared to controls in 27 patients receiving subcutaneous irradiated autologous tumor cell injections (16). In a slightly different protocol, Mahaley *et al.* vaccinated 20 glioma patients with irradiated, allogeneic glioma cell lines plus adjuvant Bacillus Calmitte-Gueurin. Although patients receiving vaccine of one cell line (U251) did have prolonged survival compared to patients receiving vaccine of another cell line (D54MG) and Karnofsky-matched historical controls, this did not reach statistical significance (129). Furthermore, only one patient developed a specific serologic response (132).

Novel Approaches to Immunotherapy

Immunogene Therapy Vaccines

With the possible exception of some forms of serotherapy still being evaluated, most glioma immunotherapies have been disappointing clinically. This has led to a decline in studies of glioma immunotherapy. However, recent advances in molecular biology and immunology have suggested novel methods to stimulate anti-tumor immunity. In particular, gene therapy is currently revolutionizing cancer immunotherapy.

Cancer immunogene therapy can be defined as transferring immunostimulatory genes to human cells in order to stimulate anti-tumor immune responses. While a detailed review of gene therapy methodology is beyond the scope of this chapter and has been performed elsewhere (262), a brief overview is in order. Like all types of gene therapy, immunogene therapy can involve *in situ* or *ex vivo* gene transfer. *In situ* therapies involve direct immunostimulatory gene transfer to tumor *in vivo*. Direct gene transfer *in situ* without the need for cell culture is advantageous but poor gene transfer efficiencies limit its applicability. *Ex vivo* therapies involve immunostimulatory gene transfer to cells *in vitro* followed by administration to patients, usually as a subcutaneous vaccine. Gene transfer is generally easier to achieve *in vitro* than *in vivo*. However, technical difficulties encountered with long-term cell culture (particularly primary cell cultures) hinder widespread application.

Many different molecular vehicles (or vectors) are available for immunostimulatory gene transfer. Genetically engineered viruses such as retroviruses, adenoviruses, herpes viruses, and adeno-associated viruses are the most common (2, 11, 100, 144). In addition, several non-viral gene transfer technologies have been developed, including liposomes, biolistics (the gene gun), calcium phosphate co-precipitation, electroporation, and even "naked" DNA injection (36, 97, 151, 252, 253). Each vector or technique has its own strengths and weaknesses.

The two most commonly used vectors to date are retroviruses and adenoviruses. Most retroviral vectors are derived from murine Maloney leukemia virus (MLV) backbones in which the *gag, pol,* and *env* genes have been deleted to ensure that viruses produced are replication-incompetent. These gene products are provided by retroviral packaging cell lines. Retroviral gene therapy vectors result in stable integration into chromosomal DNA which allows for long term *trans* gene expression with these vectors but makes them potentially mutagenic. Because most viral coat protein-encoding genes have been removed from retroviral vectors, they are relatively non-immunogenic. In contrast, adenoviruses are DNA viruses (larger than retroviruses) that allow insertion of larger *trans* genes than retroviruses. Adenoviruses do not integrate into chromosomal DNA, so they are not inherently mutagenic. However, their episomal nature means *trans* gene expression is lost relatively quickly in transfected cells. Because they retain many viral coat protein-encoding genes, they are strongly immunogenic. This further limits the duration of *trans* gene expression *in vivo*. Unlike retroviruses, adenoviral vectors can be prepared at very higher titers which improves their gene transfer efficiency.

Many cancer immunogene therapy studies have focussed on genetically engineered cancer vaccines. Based on the pioneering work of Dranoff, Jaffee, Pardoll, and others, a general strategy has evolved (10, 41, 61, 89, 249). Tumor cells from a given patient can be cultured *in vitro* and genetically modified to increase their immunogenicity. This is usually accomplished by retrovirus-mediated gene transfer (88). Genetically modified tumor cells are attenuated by irradiation to prevent further cell division and re-administered subcutaneously to the patients from whom they were derived. Numerous pro-inflammatory genes are potentially useful for immunogene therapy. These include genes encoding pro-inflammatory cytokines such as IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), or IL-12 (41, 158, 214), T cell costimulatory molecules such as B7-1 and B7-2 (76, 169, 217), and MHC molecules (166).

Successful immunogene therapy for brain tumors has been reported in several animal models. Murine studies have demonstrated that vaccination with irradiated B16-melanoma cells transduced with GM-CSF can protect against subsequent intracranial B16 challenge and prolong survival in mice with pre-established intracranial B16 tumors (192, 257). Similar results have been seen in models utilizing a murine glioma (GL261) (75). In our own laboratory, we have developed a novel model system utilizing severe combined immunodeficient (SCID) mice reconstituted with human lymphocytes to study glioma immunotherapies. Using this system, we demonstrated that growth of pre-established human glioma xenografts was markedly inhibited in allogeneic human lymphocyte-reconstituted SCID mice vaccinated with glioma cells transduced with both GM-CSF and the T cell costimulatory molecule B7-2 (163). To date, this is the only pre-clinical glioma immunogene therapy animal model utilizing human gliomas and lymphocytes.

Glioma immunogene therapy studies are not limited to transferring proinflammatory genes. Vaccination with irradiated autologous glioma cells expressing antisense molecules that inhibit glioma-derived immunosuppressive factor expression has been successful in preventing intracranial wild type tumor growth in several models. For example, vaccination with rat 9L gliosarcoma cells transfected with antisense directed at TGF-β2 markedly inhibits intracranial 9L growth (44). This result may have important clinical implications. However, a recent report has underlined the complex nature of TGF-β / glioma interactions. In a murine glioma model, glioma cells transfected with TGF-β1 inhibited anti-tumor cell-mediated immune responses, similar to human gliomas that over expressing TGF-β2. Cells transfected with TGF-β1 antisense did not have this immunosuppressive effect. These results suggest that glioma growth after TGF-β1 antisense transfection should be slower than glioma growth after TGF-β1 gene transfection due to TGF-β1's immunosuppressive effects. However, TGF-β1 expression actually inhibited tumor growth *in vivo* because it was directly toxic to glioma cells (3).

In addition to TGF-β's, other genes have been experimental targets for glioma antisense immunogene therapy. Vaccination with rat C6 glioma cells transfected with antisense directed at either insulin-like growth factor-1 (IGF-1) or insulin-like growth factor-1 receptor (IGF-1R) suppressed C6 glioma growth subcutaneously and intracranially in a $CD8^+$ T cell-dependent manner (177, 205, 219). The reason that suppressing IGF-1/IGF-1R expression in glioma cells promotes anti-glioma immune responses is not entirely clear, but it may be that blocking IGF-1 pathways upregulates MHC and T cell costimulatory (B7) molecule expression (218).

Despite the promise of vaccination with autologous genetically-modified tumor cells, there are several potential difficulties. First among these is the need to culture glioma cells from each patient in order to treat them. It is not always possible to successfully establish cultures from human gliomas (161, 243, 245). Even when cultures can be established, most early passage human glioma cultures grow slowly (much slower than established immortalized glioma cell lines) (161). This means that developing vaccines from autologous glioma cells is difficult and requires relatively long periods of time (several months). Unfortunately, this may exceed many glioma patients' life span.

Several solutions to this problem have been suggested. One of the most simple is to avoid using autologous tumor cells. In an elegant study, Ashley *et al.* demonstrated that vaccination with an allogeneic cell line transduced with a common glioma antigen (EGFRvIII) produced systemic immunity against autologous tumors (both melanomas and astrocytomas) expressing the same antigen (4). This type of "antigen-based" immunogene therapy is extremely exciting as it suggests the possibility of universal vaccines based on specific tumor antigens. It is worth noting, however, that even the most generous estimates suggest that EGFRvIII is only present in 30 -50% of malignant gliomas (246). To fully realize antigen specific therapy's potential, defining more universal tumor antigens will be necessary.

Another possibility to avoid long term glioma cultures is to use a non-tumor carrier cell to deliver immunotherapeutic gene products. This concept has been most fully developed by Dr. Roberta Glick and colleagues at Cook County Hospital in Chicago. They demonstrated that mice implanted subcutaneously or intracranially with autologous glioma cells mixed with allogeneic fibroblasts transduced with IL-2 and/or IFN-γ developed systemic anti-glioma cytotoxic immune responses and had prolonged survival (59, 122). Subsequent experiments showed that peripheral vaccination with a mixed autologous glioma cells and IL-2-transduced allogeneic fibroblasts was less effective in prolonging survival than intracranial (i.e. intratumoral) delivery of IL-2-secreting fibroblasts (60). IL-2-transduced allogeneic fibroblasts injected intracranially in mice are not detectable (even by polymerase chain reaction) after 14 days and do not induce significant toxicity (69). Using an allogeneic fibroblast to carry immunotherapuetic gene products is an attractive concept as it would allow preparation of large amounts of "vaccine" (transduced fibroblasts) without needing to culture and transduce glioma cells from individual patients.

Local Immunogene Therapy

Glioma immunogene therapy has not been limited to vaccination. Several reports have examined the effect of local cytokine production on intracranial glioma growth. The experiments reported to date involve *ex vivo* gene transfer followed by intracranial challenge with transduced cells. This is substantially different from a clinical situation where *in situ* gene transfer could be expected to result in gene transfer to far less than 100% of tumor cells. Still, they provide valuable insight into immune reactions within central nervous system tumors. In a murine glioma model, Sampson *et al.* demonstrated that mice receiving intracranial glioma cells transduced with IL-2, IL-4, or TNF-α had significantly prolonged survival compared to mice receiving wild type tumor (193). No increased survival was seen in mice receiving tumor cells transduced with interleukin-3 (IL-3), GM-CSF, or B7-1 (CD80). This is in contrast to a study by the same authors demonstrating intracranial B16 melanoma inhibition after peripheral vaccination with GM-CSF-transfected B16 cells (192) and a study by Tseng *et al.* that demonstrated marked intracranial growth inhibition for GM-CSF-transduced C6 gliomas (222). It is not clear what the source of these differences is, but it may reflect differences in tumor models. The complexity of these models is further demonstrated by the results of Fathallah-Shaykh *et al.* demonstrating prolonged survival for rats injected intracranially with IFN-γ (but not IL-2)-transduced glioma cells (46). These results conflict not only with those of Sampson *et al.* but also with the work of Glick and colleagues discussed above (59, 60, 69, 122). Although differences in tumor models in the reports published to date make it difficult to decide which immunotherapeutic gene is "best", one thing appears clear: proinflammatory cytokine expression by intracranial gliomas can result in anti-glioma immune responses that may prolong survival. Determining the most potent immunotherapeutic gene(s) for this purpose will likely require human glioma models or even clinical trials.

In addition to the studies discussed above, several reports have examined local IL-4 gene therapy for gliomas. Yu *et al.* demonstrated that both subcutaneous and intracranial human glioma growth in nude mice was inhibited if wild type cells were mixed with an IL-4-transfected murine plasmacytoma cell line (258). Similarly, Wei *et al.* demonstrated in athymic mice that rat C6 glioma growth subcutaneously or intracranially was inhibited by *in situ* retrovirus-mediated IL-4 gene transfer (236). However, because both these studies used mice that are at least partly immunodeficient, it is not clear how much of the observed anti-tumor effect is immune-mediated and how much reflects inherent IL-4 anti-tumor activity.

Other Novel Immunotherapeutic Approaches

Finally, several promising immunotherapeutic approaches that do not involve gene therapy have recently been developed. For example, work at the University of Minnesota has demonstrated that simply infusing pro-inflammatory cytokines locally around tumor cells may stimulate anti-glioma immunity. Jean *et al.* demonstrated 9L gliosarcoma rejection at distant sites following exposure to 9L cells infused subcutaneously with interleukin-12 via an osmotic minipump (91). This same group recently presented results suggesting that intracranial 9L tumor growth was inhibited in rats receiving subcutaneous vaccinations of irradiated 9L cells infused with GM-CSF and IL-2 (90). This approach has been made possible by the development of appropriate minipumps for subcutaneous implantation. Because it does not require genetic manipulation, it is substantially simpler than immunogene therapy and may be safer as well.

Another immunotherapeutic approach without genetic manipulation involves dendritic cells. Dendritic cells are bone marrow-derived cells similar to monocyte / macrophages that function as extremely potent antigen presenting cells (155, 164). These cells are such potent T cell stimulators that some investigators hypothesize that naïve T cell stimulation will only occur when antigen is presented by a dendritic cell (176). Very recent evidence suggests that dendritic cells conditioned by CD40 / CD40-ligand interactions can provide "help" (signal 2 in T cell activation, see **Figure 1-1**) directly to T_K cells without need for T_H cells (9, 181, 202). Dendritic cells exposed to tumor cells, isolated tumor antigens, or even tumor mRNA's are capable of stimulating potent anti-tumor CTL's (63). Technical advances have facilitated dendritic cell culture from peripheral blood mononuclear cells (126). All of this has led to an explosion of interest in manipulating dendritic cells to produce anti-tumor immune responses. However, few results have been published to date examining dendritic cell manipulation in glioma treatment. In one small study looking at several different therapies, Siesjo *et al.* demonstrated that immunization with tumor cells mixed with autologous dendritic cells resulted in cure for rats harboring established intracranial gliomas (208). More complete evaluation will await the further studies, but these initial results appear positive.

Clinical Results with Novel Immunotherapeutic Approaches

Novel strategies for glioma immunotherapy include immunogene therapy vaccines, local (*in situ*) immunogene therapy, vaccination with autologous tumor cells infused with cytokines, and dendritic cell manipulation. These are summarized in **Table 1-2**. Many of these strategies have yielded dramatic pre-clinical results. Based on this, several phase I clinical trials are now being developed. Presently, however, only isolated case reports have been published. Sobol *et al.* reported treating a 52 year-old woman with a glioblastoma multiforme tumor with a series of 10 subcutaneous vaccinations of autologous tumor cells transduced with IL-2 (or a mixture of wild type glioma cells and autologous fibroblasts transduced with IL-2) (209). Peripheral blood mononuclear cells from this patient showed increased CD8$^+$ cell-dependent anti-glioma cytotoxicity against autologous tumor cells after vaccination (although this was somewhat inconsistent). The patient survived for 10 months after gene therapy initiation. Ellem *et al.* reported treating a 46 year-old man with widely metastatic melanoma (including cerebral metastases) by vaccination with autologous melanoma cells transduced with GM-CSF (42). In this patient, both increased anti-melanoma delayed-type hypersensitivity reactions and increased frequency of CTL's directed against autologous melanoma were seen after vaccination. Following treatment, axillary lymph node metastases regressed, pulmonary metastases were stabilized, and a dramatic increase in cerebral edema surrounding cerebral metastases was seen. However, lesions in adrenals, pancreas, and spleen were unaffected. The patient succumbed to his disease 16 weeks after receiving his first vaccination.

Although it is impossible to make definitive conclusion based on these isolated case reports, both appear to demonstrate clinical effects for immunogene therapy vaccines, even within the central nervous system. Of note, the marked edema surrounding cerebral melanoma metastases following immunogene therapy vaccination in the case reported by Ellem *et al.* underscores some of the potential side effects of this treatment. No doubt, further study will more completely define the safety and clinical utility of these and other novel immunotherapeutic approaches in the future.

Conclusions

Malignant brain tumors, particularly gliomas, continue to be a devastating and inadequately addressed clinical problem. Immunotherapy has long been a tantalizing possibility for glioma treatment, but early approaches were largely unsuccessful. This led to a decline in interest in glioma immunology. However, modern understanding of immunology, glioma immunobiology, and molecular biology has suggested novel methods to stimulate anti-glioma immunity. This has led to renewed glioma immunotherapy investigation. Numerous studies have shown dramatic results in pre-clinical animal glioma models. Although long experience suggests that clinical results are seldom as striking as pre-clinical models, these new ways to stimulate anti-glioma immunity show great promise and will hopefully lead to effective therapies in the future.

Table 1-1

Cytokine Therapy	Serotherapy	Adoptive Transfer	Active Immunotherapy
Administering cytokines (non-specific immune adjuvants with or without direct anti-tumor activity) to promote anti-tumor immunity	Administering toxin, drug, or radioisotope-conjugated antibodies directed against tumor-specific antigens	Administering autologous lymphocytes that have been activated and expanded *in vitro*	Administering vaccines (tumor antigens, tumor cells, etc.) to stimulate systemic anti-tumor immunity

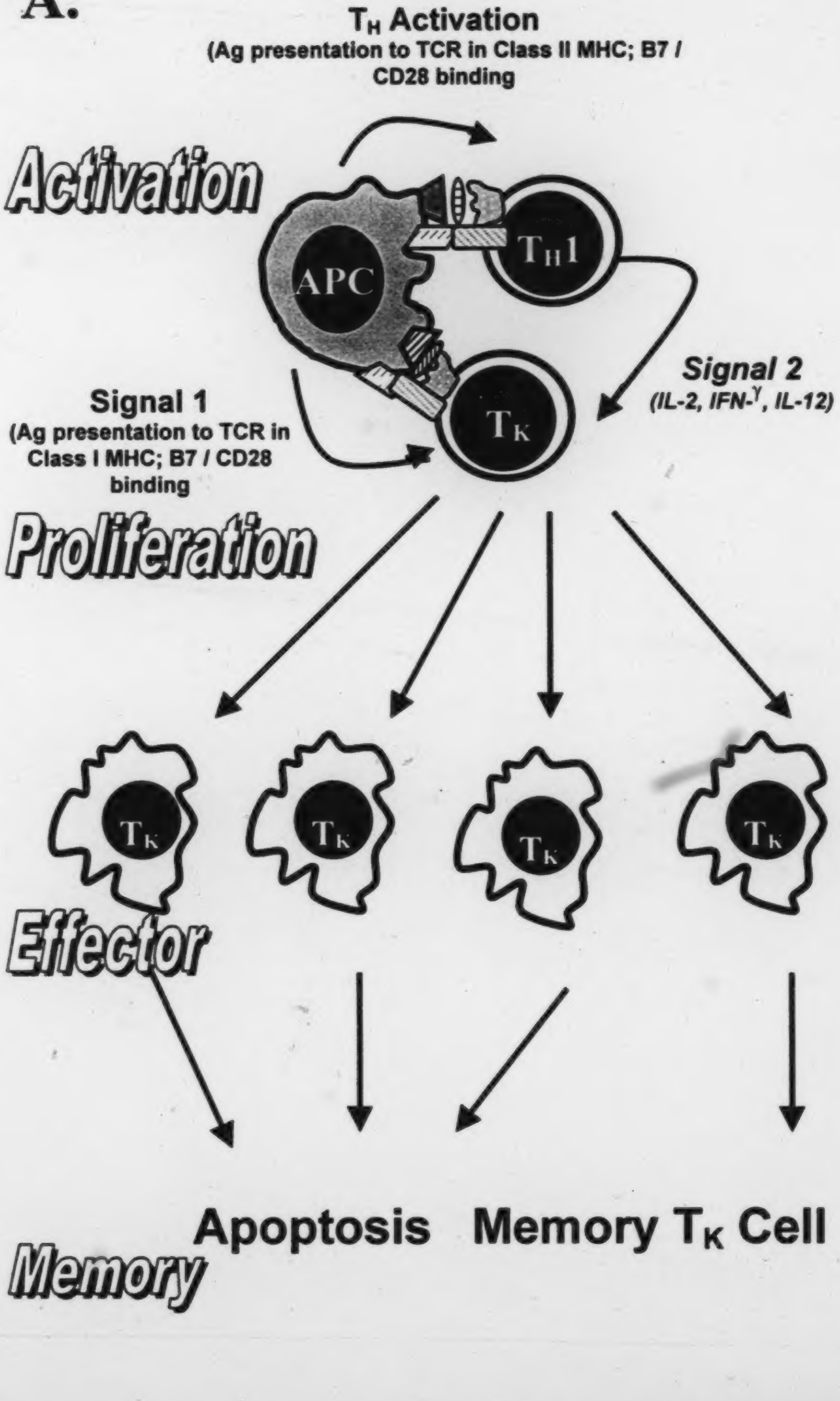
Table 1-2

Immunogene Therapy Vaccines	**Local Immunogene Therapy**	**Autologous Tumor Cell / Adjuvant Cytokine Vaccines**	**Dendritic Cell Manipulation**
Vaccination with autologous tumor cells genetically modified to enhance their immunogenicity	*In situ* proinflammatory gene transfer to tumors (or intratumoral delivery of pro-inflammatory gene-transduced carrier cells)	Vaccination with autologous tumor cells infused with pro-inflammatory cytokines via subcutaneously implanted osmotic minipumps	Vaccination with tumor antigen or mRNA-primed dendritic cells

Figure 1-1. Immune Responses

Schematic representing: A) cell-mediated immune responses, B) humoral immune responses. T_H = helper T cell. T_K = killer T cell. B = B cell. APC = antigen presenting cell. Note that both types of immune responses go through four distinct phases: activation, proliferation, effector, and memory. Recently described CD40 / CD40L pathways for APC "conditioning" to allow direct delivery of signal 2 for T_K activation are not shown.

A.


T_H Activation
(Ag presentation to TCR in Class II MHC; B7 / CD28 binding
Activation
APC
T_H1
T_K
Signal 1
(Ag presentation to TCR in Class I MHC; B7 / CD28 binding
Signal 2
(IL-2, IFN-γ, IL-12)
Proliferation
T_K
T_K
T_K
T_K
Effector
Apoptosis
Memory T_K Cell
Memory

B.

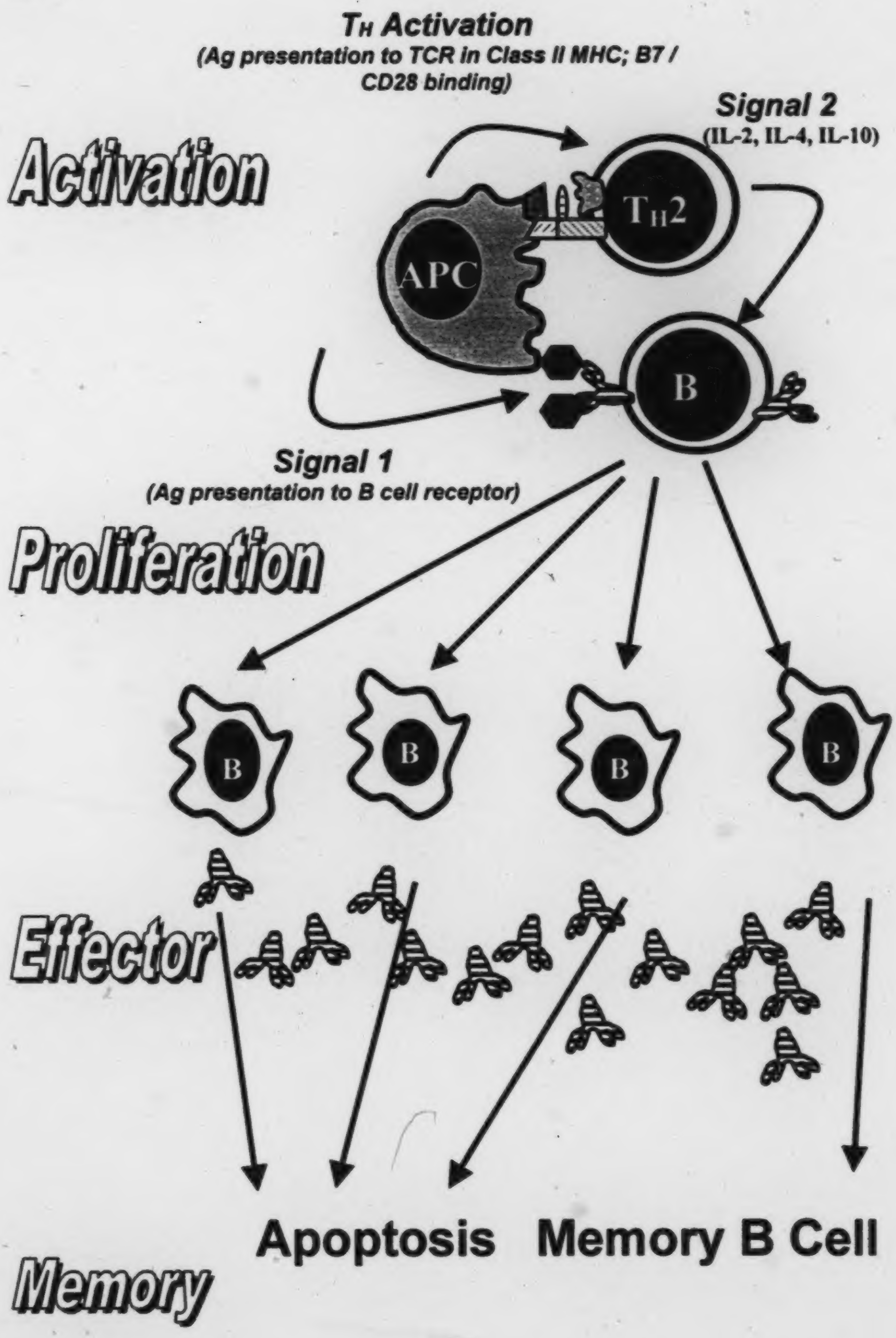
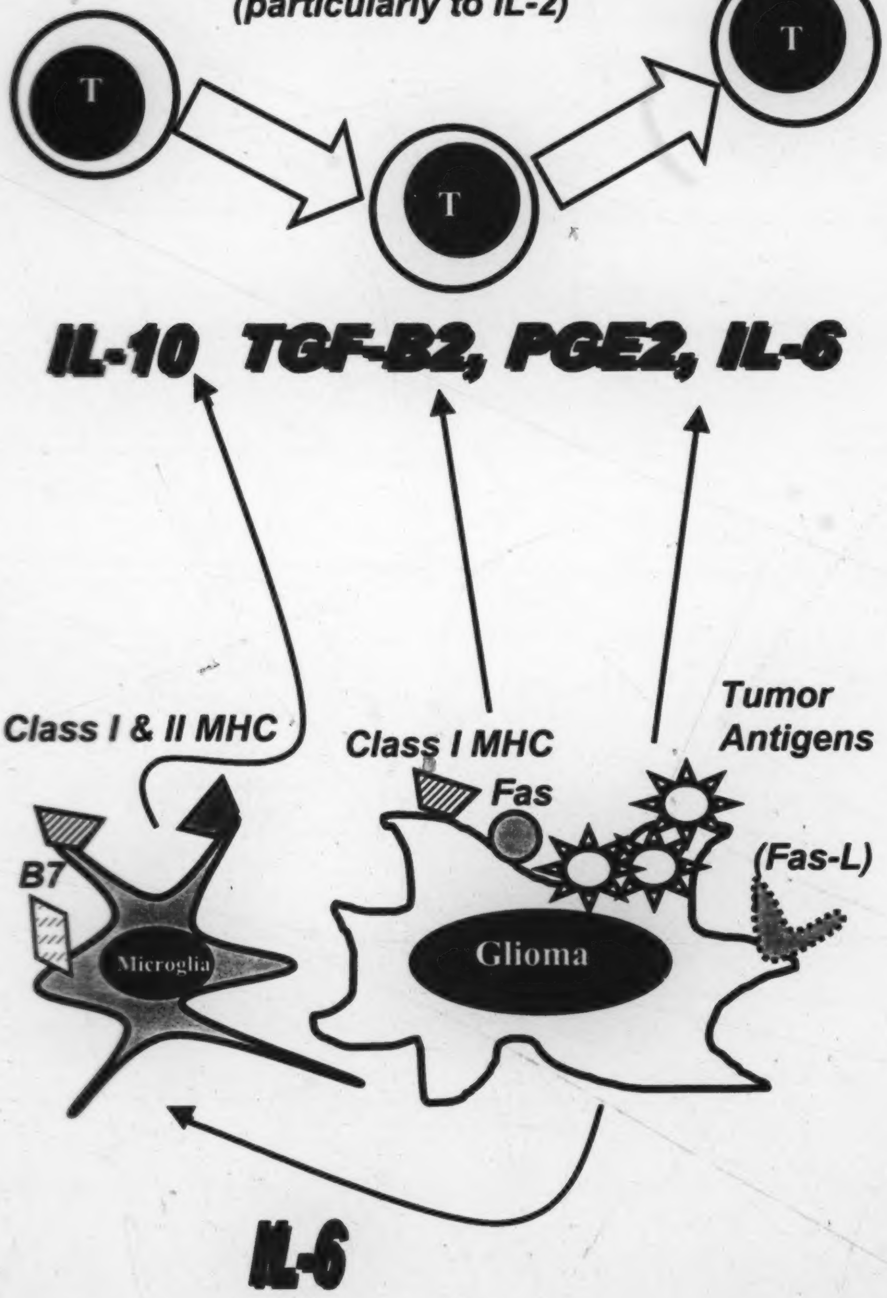

TH Activation
(Ag presentation to TCR in Class II MHC; B7 / CD28 binding)
Signal 2
(IL-2, IL-4, IL-10)
Activation
APC
TH2
B
Signal 1
(Ag presentation to B cell receptor)
Proliferation
B
B
B
B
Effector
Apoptosis
Memory B Cell
Memory

Figure 1-2. Glioma Immunobiology

Schematic representing aspects of glioma immunobiology. Gliomas appear potentially sensitive to cell-mediated immune responses but secrete several factors that inhibit lymphocyte activation. IL-2 = interleukin-2. IL-6 = interleukin-6. IL-10 = interleukin-10. TGF-B2 = transforming growth factor-β2. PGE2 = prostaglandin E2. Note that some (but not necessarily all) gliomas may express Fas-ligand.

Impaired Lymphocyte Response (particularly to IL-2)



IL-10 TGF-B2, PGE2, IL-6

Class I & II MHC

B7

Class I MHC

Fas

Tumor Antigens

(Fas-L)

IL-6

T
T
T
Microglia
Glioma

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Chapter 2

Improved Technique for Establishing Short Term Human Brain Tumor Cultures

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Introduction

Many preclinical studies of primary central nervous system (CNS) neoplasms have relied on rodent tumor models such as the rat 9L gliosarcoma and the murine C6 glioma (4, 10, 25-27). While these models have been useful, questions remain as to their validity when compared to human malignant gliomas. Human tumor models (both *in vitro* and *in vivo*) would be more ideal. However, development of animal human tumor models is dependent on efficient human CNS tumor cell growth *in vitro*.

Recent interest in glioma immunogene therapy (10, 17, 22) has underscored the need to efficiently establish at least short term human glioma cell cultures. In this novel form of *ex vivo* gene therapy, anti-tumor vaccines are created using cultured autologous human glioma cells that have been genetically modified *in vitro* to increase their immunogenicity. Clearly, successful establishment of human glioma cultures is key to this treatment strategy.

In this study, we report improved success in establishing short term human brain tumor cultures using a modified tissue processing technique. In addition, the characteristics of these cells are described in terms of morphology, immunohistochemistry, cytogenetics, and growth kinetics.

Methods

Specimen Aquistion

Eighty-seven tumor specimens (56 glioblastomas, 8 mid grade astrocytomas, 8 oligodendrogliomas, 4 metastatic adenocarcinomas, 4 melanomas, 2 meningiomas, 2 medulloblastomas, 1 pituitary adenoma, 1 lymphoma, and 1 choroid plexus papilloma) were obtained at the time of craniotomy from three hospitals from June 1988 to March 1997.

Standard Processing Technique

The first 23 samples were processed by dissection into small (< 5 mm diameter) pieces. These were incubated with gentle stirring in 0.4 mg/ml DNase (Sigma), 0.25 mg/ml collagenase IV (Gibco/BRL) and 0.5 mg/ml pronase (Boehringer-Mannheim) in Hank's buffered saline solution (HBSS) for 30 minutes at 37°C followed by 30 minutes at 4°C. This tumor slurry was passed through a tissue culture sieve and the resulting cell suspension was plated out into T25 tissue culture flasks (Corning). This protocol was kindly provided by Dr. J. Turner (Cross Cancer Institutue, University of Alberta) and has been previously shown to be highly effective in releasing cells from human tumor xenografts (1).

Modified Processing Technique

The remaining 64 samples were processed identically with the following exception: prior to plating (after filtration through the tissue culture sieve), tumor cells were layered on a density gradient media (Ficoll-Paque; Pharmacia) and centrifuged for 30 minutes at 400x g. Cells at the interface were harvested and washed twice in HBSS prior to plating. Density gradient centrifugation, as per the suggestion of Kruse, *et al.* (14), was included to remove contaminating mesenchymal cells such as fibroblasts.

Cell Culture

All cells were grown at 37°C, 5% CO_2. All cells were cultured in Dulbecco's Modified Eagle's Media / F12 (DMEM/F12) supplemented with 10% fetal bovine serum, 100 uM sodium pyruvate, 0.05 mM non-essential amino acids, 100 I.U./ml penicillin, 100 ug/ml streptomycin, and 2 mM L-glutamine (all supplements from Gibco/BRL) except those derived from melanomas. These were cultured in RPMI-1640 media supplemented as above.

Immunohistochemical Staining

Representative early passage glial tumor cultures derived from density gradient centrifuged samples underwent immunohistochemical stains using polyclonal antibodies for glial fibrillary acidic protein (GFAP) (Dako) and a monoclonal antibody for vimentin (Dako) using a commercially available immunoperoxidase kit (Vectastain ABC) as per the manufacturer's instructions. Appropriate isotype-matched control antibodies (Dako) were used for negative controls. Cells were counterstained with hematoxylin.

Karyotypic Analysis

Metaphase cells from confluent T25 flasks were harvested with 0.05% trypsin / 0.04% EDTA and replated in T75 flasks. Twenty-four to 48 hours later (when mitotic figures were abundant), colcemid (0.1 ml) was added and the flasks were incubated for a further 3 to 5 hours at 37°C. Cells were harvested with trypsin, pelleted, resuspended in a hypotonic solution, and incubated at 37°C for 15 minutes. Following this, cells were fixed with methanol / acetic acid (1:1) and placed at 4°C overnight. One to two drops of this suspension were placed on cold wet slides.. These were then incubated overnight at 60°C. Slides were briefly (5 - 10 s) immersed in a trypsin solution, then rinsed twice in PBS (pH 6.8). Finally, the slides were stained for 2 - 15 minutes in Giemsa stain, washed twice in buffered water (pH 6.8), and air dryed. Karyotypes were then read with the aid of PSE Macktype 4.6 software.

Growth Curves

Growth curves were obtained for four early passage human glioma cultures (Ed111, Ed116, Ed118, Ed120) and compared to two established human glioma cell lines (D54MG, U251) by plating 10^3 cell / well in 6 well plates. At 1 to 3 day intervals, cells from successive wells were harvested by trypsinization and counted using a hemocytometer. These experiments were performed in duplicate.

Results

The mean ages in each group (53 years for standard processing technique, 49 years for density gradient centrifugation) were similar as were the gender distributions (61% male / 39% female standard technique, 58% male / 42% female in density gradient centrifugation). Distribution of tumor types in each group is shown in **Figure 2-1**. Other than a slight increase in non-primary CNS tumors in the density centrifugation group, the spectrum of pathologies was quite similar.

Successful cultures were defined as those that survived greater than three passages in tissue culture and grew to sufficient numbers (> 10^6 cells) to allow freezing for storage. Using these criteria, the success rate was 42% (10/24) using standard processing methods. Failure was often due to outgrowth of contaminating fibroblasts as assessed by morphologic criteria (thin, spindle shaped cells) and growth characteristics (high cellular density, formation of whorls in monolayer). The success rate was 86% (55/64) with the addition of density gradient centrifugation. Success rate comparisons can be further broken down into specific tumor types as summarized in **Figure 2-2**. Not all of these cultures grew indefinitely in culture. Many appeared to die out after 15 to 20 passages. However, 20% (2/10) of cultures established using standard techniques and 17% (9/54) of cultures established with density gradient centrifugation grew for more than 20 passages without evidence of senescence.

Twenty-nine early passage human glioma cultures derived from cells prepared with density gradient centrifugation underwent immunohistochemical staining for GFAP and vimentin. Of these, 62% (18/29) stained positive for GFAP and 100% (29/29) were positive for vimentin. The mean passage number of cultures positive for GFAP (4.3 +/- 0.8) was significantly lower than the mean passage number of cultures negative for GFAP (9.1 +/- 1.5) ($P < 0.005$, Students' T test). This suggests that GFAP expression may be lost with prolonged culture. Representative stains for a glioblastoma-derived culture are shown in **Figure 2-3**.

The cellular morphology of early passage human glioma cultures is worthy of further comment. Cells appeared stellate with multiple thin processes extending from their soma. This morphology is distinct from the morphology of fibroblasts (spindle shaped cells that have few processes and tend form whorls on monolayer culture) but is similar to established human glioma cell lines such as D54MG and U251 (6). However, early passage human glioma cultures did not appear to grow to the same density as established human glioma cell lines, perhaps due to increased contact inhibition (see also **Figure 2-5**).

Karyotypic analysis demonstrated clonal abnormalities in three of the four cultures tested. Of the two oligodendroglioma-derived cultures karyotyped, Ed127 had a normal karyotype (46 X,Y) but Ed135 showed loss of a Y chromosome and gain of chromosome 7 (46 X, +7). The two glioblastoma-derived cultures that underwent cytogenetic analysis (Ed147, Ed149) both demonstrated clonal abnormalities. The Ed147 karyotype was only minimally abnormal with the loss of a Y chromosome (45 X). Ed149 demonstrated a markedly abnormal karyotype (52 XY, +X, +Y, +del (1)(p21), dic(1;2)(q25;p24), +3, -16, +18, +20, +21, +mar). These findings are summarized in **Table 2-1** and karyotypes for Ed127 and Ed149 are shown in **Figure 2-4**.

Growth curves were obtained for four representative early passage glioma cultures (Ed111, Ed116, Ed118, Ed120) and compared to two immortalized human glioma cell lines (D54MG, U251). These are shown in **Figure 2-5**. The early passage cultures demonstrated both slower growth (reduced slope of the exponential growth phase) and a higher degree of contact inhibition (plateau at a lower density) than the established cell lines.

Conclusions

Culturing human primary central nervous sytem tumor cells is not new (5, 11, 18, 19, 21, 28). However, difficulty establishing human glioma cultures has contributed to the reliance on animal CNS tumor models such as rat 9L gliosarcoma and murine C6 glioma (10, 25-27) despite significant questions regarding their similarity to human tumors (2, 4). In addition, we and others have recently proposed development of anti-glioma vaccines based on autologous tumor cells genetically modified *in vitro* to increase their immunogenicity (10, 17, 22). Successful development of such vaccines requires efficient tumor cell growth *in vitro*. Therefore, documentation and improvement of primary CNS tumor culture efficiency is important facilitation of both preclinical and clinical studies of human central nervous system neoplasms.

Although many reports have dealt with culturing human CNS tumor cells (3, 5, 6, 8, 9, 11, 13, 14, 16, 18-21, 23, 24, 28-32), few have documented the overall efficiency of establishing such cultures. One exception has been the group of Westphal, *et al.* who reported successfully cultivating more than 90% of human glioma biopsy specimens taken into culture (29) and establishing permanent cell lines in approximately 10% of these (31). This group used a technique in which bovine corneal endothelium extracellular matrix (ECM) protein-coated plates were used to initiate cultures. Although these results are impressive, evidence suggests that exposure of human glioma cells to ECM protein leads to cellular differentiation and growth inhibition (20). While such effects might be desirable for *in vivo* studies, they bring into question the usefulness of this technique for rapidly establishing human glioma cell cultures that phenotypically resemble malignant gliomas *in situ*.

In this study, we document our efficiency in establishing human brain tumor cell cultures (42%) using a standard tissue processing technique (1). By simply adding density gradient filtration centrifugation prior to plating cells (as suggested by Kruse, *et al.* (14)), we more than doubled our efficiency (86%). Improved success appeared to be due to removal of contaminating mesenchymal cells such as fibroblasts that often otherwise outgrow tumor cells. Such fibroblastic overgrowth can lead to establishment of human glioma-derived cell lines that have characteristics highly suggestive of mesenchymal origin (15, 21).

The reason that density gradient centrifugation removed fibroblasts is not immediately clear. Possibly, fibroblasts are not as readily dissociated into single cells by the steps preceding the density gradient centrifugation resulting in heavier clumps of fibroblasts being pelleted while tumor cells in a single cell suspension accumulate at the interface of the density gradient. Unfortunately, it is difficult to test this hypothesis directly as necrotic debris and residual red blood cells found in pellets after density gradient centrifugation obscure attempts at careful immunohistochemical analysis. Other methods have been proposed to eliminate fibroblasts from early CNS tumor cultures including serum starvation for the first 2 to 3 days *in vitro*. Although our experience with this technique is limited, our results to date have been uniformly negative (data not shown).

The purpose of this study is to outline an improved technique for establishing short term human brain tumor cultures. This is not the same as establishing immortalized cell lines. Many of our cultures died out after 15 to 20 passages. Nevertheless, 20% (2/10) of cultures established using standard techniques and 17% (9/54) of cultures established with density gradient centrifugation grew for more than 20 passages without evidence of senescence. This is slightly higher than the proportion of immortalized cell lines derived from short term glioma cultures previously reported (31). However, some of these cultures may still die out with prolonged passage.

Immunohistochemical staining was performed to characterize early passage glioma cultures. The fact that these glial tumor-derived cells all appear to express vimentin and most (62%) express GFAP is consistent with their glial origin (3, 5, 14, 21, 23, 24, 32). It might be suggested that cultures without GFAP expression may represent outgrowth of non-neoplastic mesenchymal passenger cells that were not removed by density gradient centrifugation. While we cannot absolutely exclude this possibility, the morphology of these cells (stellate, low density) mirrors the morphology of their GFAP-positive counterparts and is markedly different from the morphology of expected contaminating cells such as fibroblasts (spindle shaped, high density). Furthermore, the mean passage number for GFAP positive cultures (4.3 +/- 0.8) is significantly lower ($P<0.01$) than the passage number for GFAP negative cultures (9.1 +/-1.5). This suggests that GFAP negative cultures more likely represent neoplastic glial cells that have lost GFAP expression with prolonged growth *in vitro*. Such loss of GFAP expression in cultured human glioma cells has been well documented previously (8, 13, 32).

Cytogenetic analysis was undertaken on four representative human glioma-derived early passage cultures. These results are summarized in **Table 2-1**. One oligodendroglioma culture (Ed129) demonstrated clonal gain of chromosome 7 and loss of a Y chromosome (46 X, +7; **Figure 2-4A**) while the other oligodendroglioma culture (Ed135) had a normal karyotype (46 X, Y). Although more complex clonal cytogenetic abnormalities have been previously reported in some human oligodendrogliomas, it is not unusual to see simple clonal or normal karyotypes in these tumors (12). It is interesting to note that the Ed129 culture demonstrated gain of chromosome 7. The epidermal growth factor receptor (EGFR) gene (erb-B1) has been localized to this chromosome and overexpression of EGFR by gliomas has been associated with gain of chromosome 7 (30).

The karyotypes of the two glioblastoma-derived cultures tested were both abnormal. Ed147 had a simple clonal abnormality with loss of a gonosome (45 X) while Ed149 had a complex clonal abnormality (52 XY, +X, +Y, +del (1)(p21), dic(1;2)(q25;p24), +3, -16, +18, +20, +21, +mar) (**Figure 2-4B**). Although cytogenetic findings in human glioblastomas can reflect a wide spectrum of abnormalities that include similar findings to these cells (12), many of the most commonly reported chrosomal abnormalities in human glioblastomas (+7, -10, -13, -17) (3, 7, 12, 23, 30) are not present and the gain of a marker chromosome is relatively unusual. Nevertheless, the cytogenetic findings in these glioblastoma cultures are consistent with their malignant glial origin.

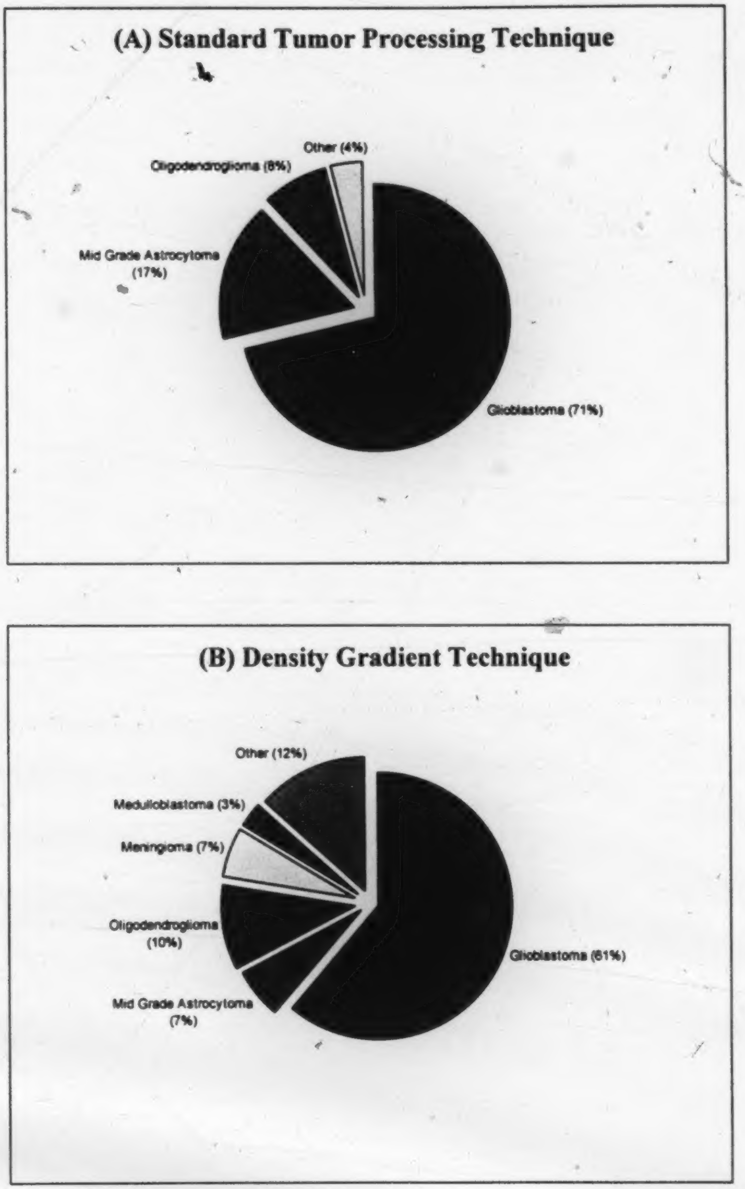
Finally, *in vitro* growth curves of early passage cultures showed that they appear to grow slower and with a higher degree of contact inhibition than immortalized human glioblastoma cell lines (**Figure 2-5**). This growth pattern has important implications for experimental work with these cultures. Immortalized cell lines either represent cultures with higher inherent growth potentials or cultures that have mutated to allow greater growth potential. If early passage cultures are carried long enough *in vitro*, a minority may establish immortalized cell lines (29, 31). In the short term, however, experimental protocols using human CNS neoplasm early passage cultures should reflect their slower growth rates compared to immortalized cell lines. This is particularly germane to immunogene therapy protocols in which genetically modified autologous tumor cells are used as the basis for vaccines (10, 17, 22).

The technique described in this report is highly efficient for establishing short term human CNS tumor cultures and characterization of these cells is consistent with their tumors of origin. This should aid in the development of human CNS tumor models for preclinical investigations and in clinical protocols dependent upon growth of human CNS tumor cultures. However, the slow growth rate of these cells compared to immortalized cell lines must be recognized and incorporated into any such experimental protocols.

Glioma Culture	*Pathology*	*Karyotype*	*# Cells*	*Comment*
Ed129	Oligodendroglioma	46, XY	1	Simple Clonal
		46, X, +7	8	
		46, XY, +7, -20	1	
Ed135	Oligodendroglioma	46, XY	8	Simple Non-Clonal
Ed147	Glioblastoma	46, XY	1	Simple Clonal
		45, X	4	
		44, X, -8	1	
		45, XY, -22	1	
		59, XY	1	
Ed149	Glioblastoma	52, XY, +X,+Y, del(1)(p21), dic(1;2)(q23;p24), +3, -16, +18, +20, +21, +mar	7	Complex Clonal
		51, XY, +X,+Y del(1)(p21), dic(1;2)(q23;p24), +3, -8, -16, +18, +20, +21, +mar	1	
		50, XY, del(1)(p21), dic(1;2)(q23;p24), +3, -16, +18, +20, +21, +mar	1	
		51, XY, +X,+Y, del(1)(p21), dic(1;2)(q23;p24), +3, -16, +18, +20, +21, -22, +mar	1	

Table 2-1. Cultured Human Glioma Karyotype Analysis

Summary of karyotypic analysis of four short term glial tumor cultures. "Number of cells" refers to the number of cells tested that had a particular karyotype.


(A) Standard Tumor Processing Technique

(B) Density Gradient Technique

Figure 2-1. Pathology by Processing Technique

Relative frequency of pathologic diagnoses in samples processed by standard technique (A) and with the addition of density gradient centrifugation (B).

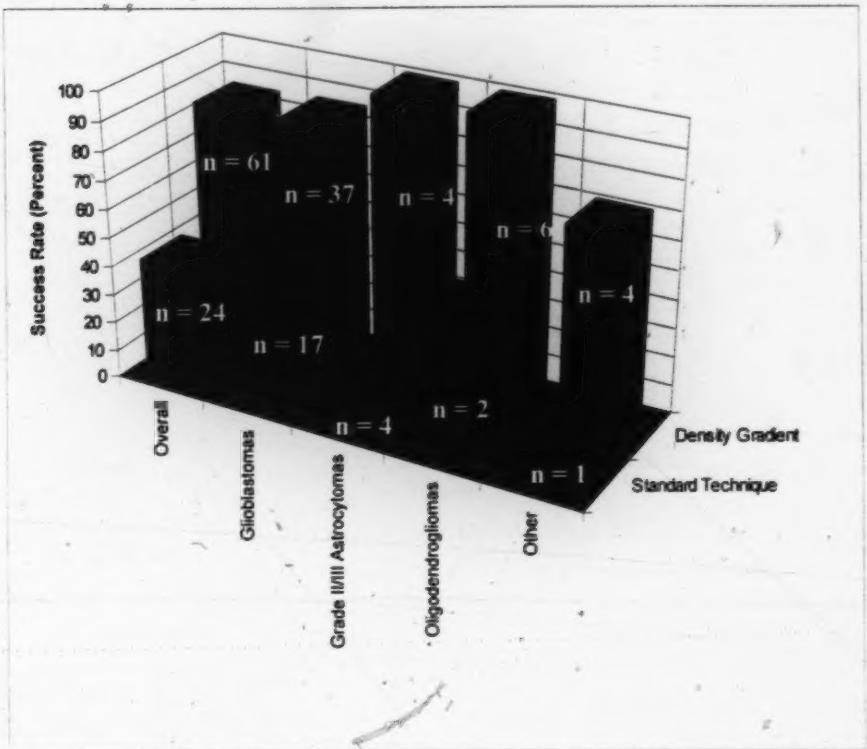


Figure 2-2. Culture Success Rates

Success rates in establishing human brain tumor cultures using a standard processing technique (dark gray) and with the addition of density gradient centrifugation (light gray). Combined (total) efficiency rates are shown on the far left while success rates by tumor type are shown to the right.

Figure 2-3.

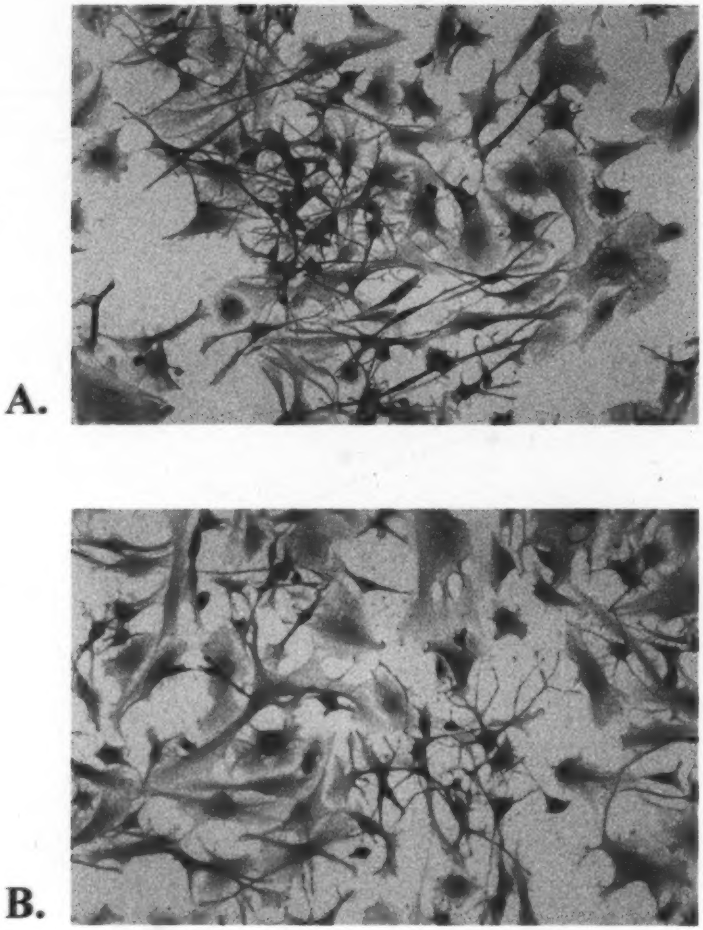


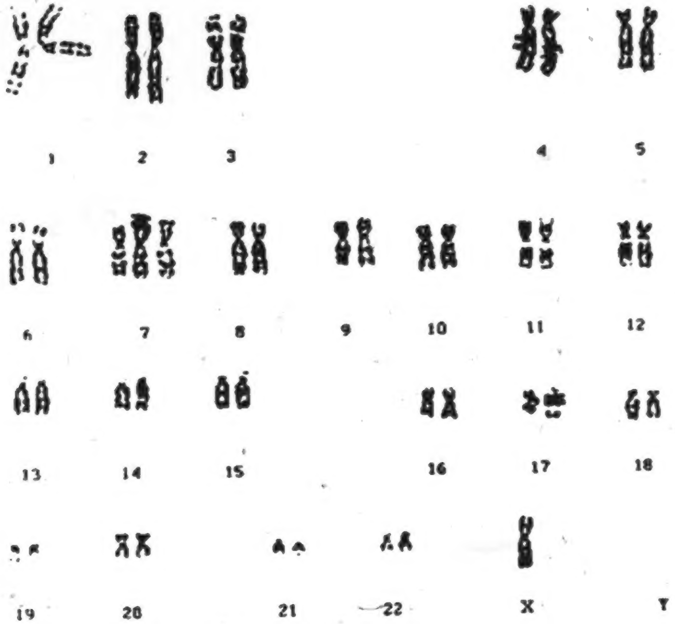
Figure 2-3. GFAP and Vimentin Expression

Immunohistochemical staining of the human glioblastoma multiforme culture Ed147 (520x). A) GFAP; B) vimentin. Brown color represents positive staining.

Figure 2-4. Representative Human Glioma Karyotypes

A) Representative Ed129 (early passage human oligodendroglioma culture) karyotype showing simple clonal abnormalty (46 X, +7). B) Representative Ed149 (early passage human glioblastoma culture) karyotype showing complex clonal abnormality (52 XY, +X, +Y, +del (1)(p21), dic(1;2)(q25;p24), +3, -16, +18, +20, +21, +mar).

A).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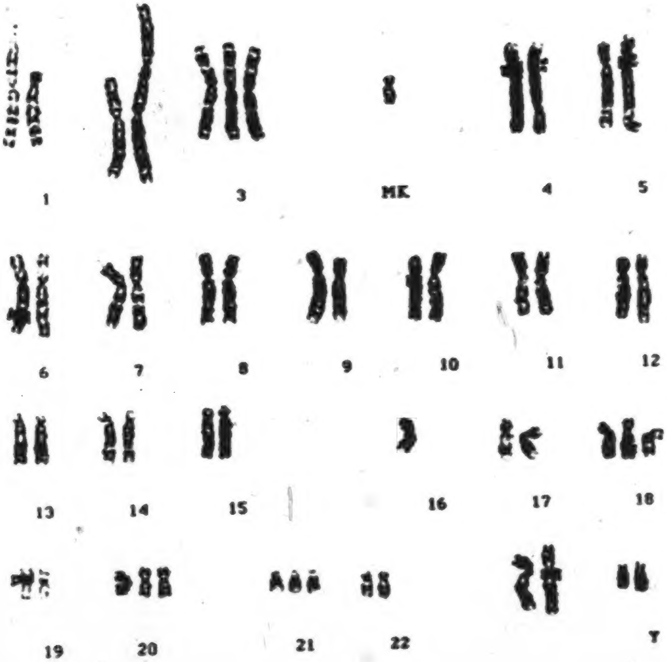
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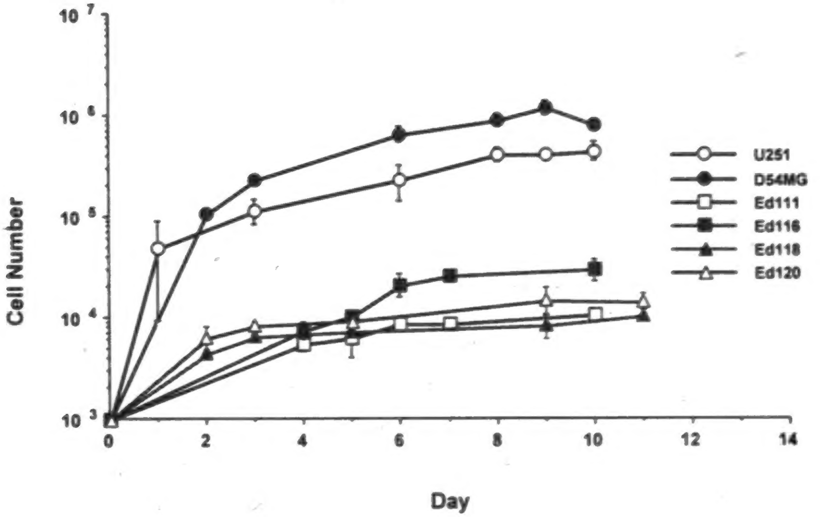

Figure 2-5. Cultured Human Glioma Growth Curves

Growth curves for two immortalized human glioblastoma cell lines (D54MG, U251) and four early passage human glioma cultures (Ed111, Ed116, Ed118, Ed120). Error bars represent standard deviations.

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Chapter 3

Human Glioma Immunobiology *In Vitro*: Implications for Immunogene Therapy

A form of this chapter has been submitted for publication. Parney et al. 1999. Neurosurgery Submitted.

Introduction

Despite intensive efforts, primary malignant gliomas such as glioblastoma multiforme continue to be resistant to treatment. Mean survival is less than one year (6, 15). Therefore, identifying useful therapeutic alternatives is vital. One such alternative, glioma immunotherapy, is particularly attractive as it has the potential to specifically target tumor cells while leaving normal tissues unharmed (28). However, glioma-induced immunosuppression has significantly hindered attempts at immunotherapy (65).

Tumor-derived immunosuppression has a number of known pathways. Some tumors downregulate class I and II major histocompatibility complex (MHC) expression, rendering them poor antigen presenting cells for T lymphocytes (14, 47). This has been proposed as a mechanism of immunosuppression in gliomas (33). In addition, gliomas have been reported to express immunoinhibitory factors such as transforming growth factor-β2 (TGF-β2) (4, 31, 57) and prostaglandin E2 (PGE2) (16, 54). Gliomas may also secrete interleukin-6 (IL-6) (37, 63) and interleukin-10 (IL-10) (25, 27, 43). IL-6 and IL-10 may have the ability to shift anti-glioma immune responses to T_H2 (humoral) pathways that are less effective at eradicating tumor then cell-mediated (T_H1) immune responses.

To counteract glioma-derived immunosuppression, recent immunotherapy studies have focussed on genetically modifying glioma cells to enhance their immunogenicity. This is known as immunogene therapy. Our laboratory (45) and others (13, 21, 52) have reported successful glioma immunogene therapy in pre-clinical animal models.

Immunogene therapy assumes that, despite glioma-derived immunosuppression, cytotoxic lymphocytes activated by genetically modified tumor cells will be able to kill wild type glioma cells. While early results in animal studies appear to support this premise, the molecular correlates of this assumption are not entirely clear (particularly in human gliomas). Furthermore, different tumor models have produced conflicting results concerning immunogene therapy strategies. For example, Dranoff *et al.*, Sampson *et al.*, and Yu *et al.* have all reported that vaccination with GM-CSF-transduced B16 melanoma cells induces potent and long-lasting anti-B16 immunity that can eradicate intracranial tumors in syngeneic animals (12, 52, 68). However, when Samson *et al.* examined immunogene therapy in a syngeneic murine glioma model, they found that GM-CSF-transduced glioma cells were relatively ineffective vaccines compared to IL-2, interleukin-4 (IL-4), or tumor necrosis factor-α (TNF-α)-transduced cells (53). In our laboratory, using a human glioma model, we observed moderate growth inhibition for gliomas transduced with GM-CSF (45).

Because of these conflicting results, it is difficult to predict which immunogene therapy strategy (i.e. which immunostimulatory gene) will have the most impact on human gliomas. Different responses in different animal models likely reflect variations in tumor immunobiology. Accurately predicting human glioma responsiveness to immunogene therapy will require an improved understanding of human glioma immunobiology. Achieving this understanding may help evaluate the validity of the molecular assumptions underlying glioma immunogene therapy (namely, that human gliomas are potentially sensitive to immune responses despite a lack of anti-tumor immunity in glioma patients clinically). Therefore, we examined the expression of 10 immunologically important molecules (Class I and II MHC, B7-2, Fas, IL-6, IL-10, IL-12, TGF-β2, PGE2, and GM-CSF) by human glioma cells *in vitro* (primarily early passage cultures) in order to help clarify these issues. Early passage cultures appear to be the most suitable template for immunogene therapy vaccines, so establishing their baseline immunologic expression patterns is particularly relevant.

Methods

Human Glioma Cell Culture and Cell Lines

Early passage cultures were established from fresh operative tumor samples as previously described (44). Briefly, samples were minced into small (1 - 2 mm^3 diameter) pieces and incubated with gentle stirring in 0.4 mg/ml DNase (Sigma), 0.25 mg/ml collagenase IV (Gibco/BRL) and 0.5 mg/ml pronase

(Boehringer-Mannheim) in Hank's buffered saline solution (HBSS) for 30 minutes at 37°C followed by 30 minutes at 4°C. This tumor slurry was passed through a tissue culture sieve. Prior to plating, tumor cells were layered on a density gradient media (Ficoll-Paque; Pharmacia) and centrifuged for 30 minutes at 400x g. Cells at the interface were harvested and washed twice in HBSS then plated in T25 flasks (Corning). Early passage cultures were grown at 37°C, 5% CO_2 in Dulbecco's Modified Eagle's Media / F12 (DMEM/F12) supplemented with 10% fetal bovine serum, 100 uM sodium pyruvate, 0.05 mM non-essential amino acids 100 I.U./ml penicillin, 100 ug/ml streptomycin, and 2 mM L-glutamine (all supplements from Gibco/BRL).

The D54MG and U251 cell lines were graciously provided by Dr. D. Bigner (Duke University) and Dr. R. Day (Cross Cancer Institute, University of Alberta), respectively. They were grown at 37°C, 5% CO_2 in DMEM (not DMEM/F12) media supplemented as above.

Flow Cytometry

Established cell lines (D54MG, U251) and early passage (< 10 passages) glioma cultures (Ed77 - Ed149) were grown in T150 flasks until they reached ~70% confluence. Cells were harvested by incubation in 2 ml 0.4% EDTA in phosphate-buffered saline (PBS) for 30 minutes at 37°C followed by repeated pipetting. These cells were pelleted (5000 rpm x 5 minutes, 4°C), washed once in immunofluourescence (IF) buffer (2% fetal calf serum, 0.02% sodium azide in PBS), resuspended in IF buffer, and placed on ice.

Aliquots of 200 µl were placed in 1.5 ml Eppendorf tubes and incubated on ice, in the dark, for one hour with the following antibodies (individually): 1 ul anti-class I MHC-FITC (Pharmingen), 1 ul anti-Class II MHC-FITC (Pharmingen), and 1 ul anti-B7-2-RPE (Ancell). Aliquots were also incubated with appropriate isotype- and fluourochrome-matched antibodies (Dako). After incubation, cells were pelleted (2000 rpm x 5 minutes) and washed three times in IF buffer. Finally, cells were resuspended in 0.5 ml of 1% formalin in PBS.

Staining for Fas proceeded somewhat differently. Aliquots of 200 µl of cells were placed in 1.5 ml Eppendorf tubes and incubated on ice for one hour with either 1 µl of mouse anti-Fas (Pharmingen) or an isotype-matched control mouse antibody (Dako). Following this primary incubation, cells were washed three times in IF buffer and resuspended in 200 µl of 10% normal goat serum (Dako) in IF buffer. Samples were incubated for 10 minutes at room temperature then immediately (without washing) incubated in the dark, on ice, for one hour with 1 µl of goat anti-mouse Ig-FITC (Pharmingen). Samples were washed three times in IF buffer and resuspended in 0.5 ml of 1% formalin in PBS.

After staining, samples were read on a Becton-Dickison flow cytometer using either Lysis II or CellQuest 2.0 software. Histograms were obtained on the FL-1 parameter (class I MHC, class II MHC, Fas, and controls) or the FL-2 parameter (B7-2 and controls).

ELISA's for Cytokine Expression

Established cell lines (D54MG, U251) and 24 early passage (< 10 passages) glioma cultures (Ed77 - Ed149) were grown in T75 flasks until they reached ~70% confluence. Fresh media was supplied (10 ml) and harvested after incubation for 24 hours. After centrifuging (5000 rpm x 5 min) to remove cells and debris, the conditioned media was aliquoted into 500 μl samples and stored at -80°C until ELISA's were performed. Cells from these T75 flasks were harvested with trypsin, resuspended in appropriate growth media, and counted on a hemocytometer with trypan blue viability staining.

Enzyme-linked immuno-absorbant assays (ELISA's) of the conditioned media were performed using commercially available kits (IL-6, IL-10, IL-12, GM-CSF, TGF-β2: R + D Systems; PGE2:Cayman Chemical) as per the manufactures' instructions. All ELISA kits measured cytokines to the level of picograms per millilitre. For TGF-β2, two other considerations were important. TGF-β2 in test samples was activated by adding 10 μl of 0.1 N HCl to 200 μl of conditioned media prior to performing the ELISA. In addition, fetal bovine serum (FBS) used to supplement culture media resulted in significant background TGF-β2 levels. Levels were determined from fresh, FBS-supplemented media and were subtracted from TGF-β2 values from glioma-conditioned media. All ELISA's were performed in triplicate.

Results

The tumor code, age, sex, and pathology of patients from whom early passage glioma cultures were derived are summarized in **Table 3-1**. The average age was 45 + / - 14 years. Fifty-eight percent (14/24) were male and 42% were female. The vast majority of patients suffered from glioblastomas (21/24). Two patients had anaplastic astrocytomas (grade III) and one patient had an anaplastic oligodendroglioma.

All samples tested (16/16; the cell lines D54MG and U251 and 14 early passage cultures) were positive for class I MHC by flow cytometry. Only 1 of 16 samples tested (Ed147) expressed class II MHC. No samples (0/16) expressed B7-2. All samples (16/16) stained positive for Fas. **Figures 3-1 to 3-4** show representative histograms for class I MHC, class II MHC, B7-2, and Fas respectively. Flow cytometry is difficult to quantify, as staining is essentially "positive" or "negative." However, there was some variability in the degree of shift to the right for some "positive" histograms, particularly for class I MHC (**Figure 3-1**), suggesting some variability in expression intensity. Results of flow cytometry staining are summarized in **Table 3-1**.

Culture supernatant cytokine concentrations (as measured by ELISA) were converted to ng/10^6cells/day using the following equation:

$$\text{Cytokine Production} = \frac{[\text{Supernatant}] \times \text{Total Volume (10 ml)} \times 10^6}{\text{Time (1 day)} \times \text{Total Cell Number}}$$

Nearly all glioma cultures expressed high levels of IL-6 (19/21; mean + / - s.e.m. = 36.537 + / - 10.831 ng/10^6cells/day) and PGE2 (21/21; 15.607 + / - 4.554 ng/10^6cells/day). Sixty-two percent (13/21; 8.681 + / - 3.789 ng/10^6cells/day) expressed TGF-β2. Forty-three percent (6/14) expressed measurable GM-CSF but expression levels were low (0.218 + / - 0.121 ng/10^6cells/day). Few expressed measurable IL-10 (4/21; 0.171 + / - 0.092) and none expressed IL-12 (0/22). By student T test, mean IL-6, PGE2, and TGF-β2 levels were all significantly higher than IL-10, IL-12, or GM-CSF ($p < 0.001$ for each). In addition, IL-6 levels were significantly higher than PGE2 ($p < 0.05$) and TGF-β2 ($p < 0.01$). For each cytokine, two samples tested were from established cell lines (D54MG, U251) while the remainder were from early passage cultures. **Figure 3-5** shows a scatter graph of cytokine expression by glioma cells *in vitro*. Cytokine expression is summarized in **Table 3-1**.

Conclusions

Previous studies have uncovered numerous immunosuppressive pathways in gliomas blocking attempts at immunotherapy (28, 33, 65). However, recent advances have suggested novel strategies to stimulate anti-glioma immunity, including immunogene therapy (12, 13, 20). Unfortunately, results in pre-clinical studies have varied considerably in different tumor models, possibly due to differences in tumor immunobiology (45, 52, 53, 68) making prediction as to which immunogene therapy strategy will be most effective against human gliomas difficult. Therefore, we wished to examine aspects of human glioma immunobiology in order to gain insight into appropriate strategies for glioma immunotherapy. We also wanted to establish whether or not molecular correlates exist in human gliomas for the central assumption in immunogene therapy: that human gliomas are potentially sensitive to immune responses, despite the innate suppression of effective anti-tumor immunity in glioma patients.

Early passage glioma cells have been proposed as an appropriate template for immunogene therapy vaccines (13, 45, 52). Because of this, we based our immunobiologic studies primarily on early passage human glioma cultures. However, care must be taken in extrapolating these results to expression patterns *in situ* as culture results may not reflect all the conditions found within the tumor microenvironment. Nevertheless, this data reflects aspects of human glioma immunology that may be useful in designing glioma immunotherapies.

Human glioma MHC expression is controversial. Based on immunohistochemical analysis of glioma sections, some authors report that most gliomas express class I MHC and a significant minority express class II MHC (39, 51) while others report little MHC expression at all (34, 42). Expression *in vitro* has been similarly controversial (9, 11, 58). Lampson and colleagues found that most gliomas are negative for class I and II MHC and argued that variable glioma MHC staining results in the literature can largely be explained by variations in antibodies, controls, and *in vitro* / *in vivo* conditions (32-34). Our present study, which demonstrated an overwhelming class I MHC$^+$ / class II MHC $^-$ status for cultured human gliomas by highly sensitive flow cytometry (**Figures 3-1 and 3-2, Table 3-1**), may add to this controversy. However, it may be possible to explain the discrepancies between our results and Lampson *et al.*'s by simple differences in the sensitivities of our respective MHC expression assays.

Class I MHC may be expressed by gliomas at relatively low levels that could be missed by routine immunohistochemistry. In contrast, flow cytometry is highly sensitive and may be demonstrate expression even if levels are too low for immunohistochemical detection. Low level background expression is supported by Lampson's finding that class I MHC expression can be upregulated in gliomas after interferon-γ (IFN-γ) exposure (33). In tumors that completely lose class I MHC expression (such as many melanomas), β_2-microglobulin gene deletions/mutations are usually present that completely prevent class I MHC expression, even after IFN-γ exposure (14, 47). Therefore, easily detectable class I MHC expression in gliomas after IFN-γ exposure suggests that low-level background expression was present prior to IFN-γ treatment. This is consistent with our results. Class I MHC expression (albeit, possibly at low levels) is promising for immunotherapy as it suggests gliomas can present antigen to CD8+ cytotoxic T cells.

Many factors in addition to MHC expression contribute to the ability to stimulate immune responses. T cell costimulatory molecules such as B7-1 and B7-2 have an increasingly recognized role in T cell activation (1). In addition to antigen presentation in the context of MHC, it appears that a second "costimulatory" signal (B7-1 or B7-2 binding to CD28 on the T cell surface) is necessary for complete T cell activation and to prevent T cell anergy (7, 17, 38, 55, 67). Initial work suggested that B7-1 might be more important in stimulating T_H1 (cellular) immune responses while B7-2 caused a shift to T_H2 (humoral) pathways (30, 60). However, further studies have questioned this conclusion and suggested that B7-2 may be a more important physiologically (19, 30, 35, 46).

Lack of T cell costimulation is a potential mechanism for tumor cells to avoid immune surveillance (8). Trojan *et al.* have reported that B7 expression was absent in rat C6 glioma and a small number of human glioma primary cultures but could be upregulated by transfecting insulin-like growth factor-1 (IGF-1) antisense cDNA (61). In our study, 0/16 samples showed evidence for B7-2 expression (**Figure 3-3, Table 3-1**). T cells from glioma patients characteristically exhibit a high degree of anergy (48). Given that gliomas do not appear to express B7 molecules, one can speculate that lack of T cell costimulatory signals may contribute to this anergy.

Even if anti-tumor lymphocytes are activated, gliomas may not be sensitive to cell-mediated immunity. Sensitivity to cytotoxic T cell-mediated killing requires expression of other factors, including Fas (a member of the tumor necrosis factor-receptor superfamily). Fas^+ cells undergo apoptosis (programmed cell death) when they come in contact with Fas-ligand (FasL) (36). The Fas / FasL interaction is felt to be the primary pathway that activated cytotoxic ($CD8^+$) T cells utilize to kill their targets (29). Other pathways such as the perforin /

granzyme pathway also may be involved (3, 10), but these pathways are dependent on supraphysiologic Ca^{++} levels which limits their importance *in vivo* (49).

Weller and colleagues have previously reported Fas expression in four of seven human glioma cell lines (66). In our present study, 16/16 human glioma cultures were Fas^+ (**Figure 3-4, Table 3-1**) suggesting that malignant glial cells may be sensitive to FasL-mediated apoptosis. However, recent reports also suggest that some gliomas are $FasL^+$ (22, 50) which suggests insensitivity to FasL-mediated apoptosis. In addition, FasL expression may further enhance immunosuppression by inducing apoptosis in naïve (Fas^+) T cells (23). Despite these findings, several groups have reported that Fas^+ glioma cells undergo apoptosis after exposure to FasL or agonistic anti-Fas antibodies (2, 64, 66). Therefore, it is reasonable to conclude that at least some glioma cells (perhaps Fas^+ / $FasL^-$) are sensitive to Fas / FasL-mediated apoptosis.

Many cytokines have the ability to influence anti-tumor immune responses. Expression of immunosuppressive cytokines such as PGE2 and TGF-β2 by human gliomas has been reported previously (4, 16, 31, 54, 57). Our results (**Figure 3-5, Table 3-1**) are consistent with TGF-β2 and PGE2 playing an important immunosuppressive role in the majority of gliomas.

Both IL-6 and IL-10 expression has been previously reported in human gliomas (25, 27, 37, 43, 63). While high IL-6 expression in 19/21 glioma cultures we examined (mean 36.537 ng/10^6 cells/day) is consistent with these findings, minimal IL-10 expression (4/21 samples; mean 0.173 ng/10^6 cells/day) is not. Most previous reports of IL-10 expression by gliomas have been based on total RNA analysis from fresh glioma specimens using reverse transcriptase polymerase chain reaction (rtPCR) (25, 27, 43). Using rtPCR, it is not possible to differentiate between different cellular mRNA sources. Therefore, for fresh glioma specimens, it is not possible to know whether glioma cells themselves were responsible for IL-10 mRNA or whether this mRNA was derived from other cells such as infiltrating microglia or lymphocytes. Indeed, Huettner *et al.* demonstrated through *in situ* hybridization studies that a large portion of glioma-derived IL-10 mRNA localizes microglia (26). In light of these findings, minimal IL-10 expression by our cultured gliomas is not surprising. Interestingly, it has been reported that IL-6 exposure can stimulate IL-10 secretion by microglia *in vitro* (56). Therefore, one can speculate that glioma-derived IL-6 may stimulate microglial IL-10 expression *in vivo*.

Both IL-6 and IL-10 are T_H2 cytokines that shift immune responses to humoral immunity (41). Humoral immune responses are likely to be less effective at eradicating solid tumors like gliomas than cell-mediated cytotoxic (T_H1) responses due to poor antibody penetration into tumors *in situ*. It is possible that glioma and/or microglia-derived IL-6 and IL-10 shift glioma-infiltrating T cells to T_H2 pathways. T_H2 shifts may be further augmented by glioma-derived PGE2, which inhibits production of IL-12 (a potent T_H1 cytokine) (62). Consistent with these observations, Brooks *et al.* have recently reported a T_H2 shift in peripheral blood mononuclear cells exposed to glioma culture supernatants (5).

Given that gliomas are generally immunosuppressive, minimal or absent expression of proinflammatory cytokines (GM-CSF, IL-12) is not surprising. GM-CSF is known to stimulate antigen presenting cells such as dendritic cells and microglia (24, 69). Low level GM-CSF expression by gliomas *in vitro* but not *in vivo* has been reported previously (18). Our results are consistent with this finding. IL-12 expression levels in gliomas have not been reported previously. Given that this cytokine could potentially stimulate effective anti-tumor immune response by stimulating natural killer lymphocytes and shifting immune responses to T_H1 pathways (59), its absence in glioma cultures is not surprising. This is particularly true considering that glioma-derived PGE2 may inhibit IL-12 expression (62).

This study allows comparison between different glioma-derived cytokine expression levels. Pro-inflammatory cytokines such as IL-12 and GM-CSF that are minimally expressed likely have little impact on glioma biology. However, cytokines such as IL-6, PGE2, and TGF-β2 that are significantly expressed may be important modifiers of glioma immunology. In our study, cultured gliomas secreted considerably more IL-6 (mean 36.537 ng/10^6cells/day) than PGE2 (mean 15.607 ng/10^6cells/day; $p < 0.01$) or TGF-β2 (mean 8.681 ng/10^6cells/day; $p < 0.05$). If the relative potencies of these cytokines are similar, this suggests that IL-6 may play a greater role in glioma immunology than either PGE2 or TGF-β2 and therapies directed at IL-6 would be expected to be more potent than those directed at PGE2 or TGF-β2.

The present study further clarifies some aspects of human glioma immunobiology. Class I MHC and Fas expression suggests that gliomas can be recognized and killed (respectively) by cytotoxic CD8+ T lymphocytes. However, because human glioma cells are class II MHC $^-$ and B7-2 $^-$, they are not ideal antigen-presenting cells. Furthermore, PGE2 and TGF-β2 secretion suggests that human glioma cells exert potent immunosuppressive effects. This may be augmented by IL-6 expression that can shift anti-tumor immunity to ineffective T_H2 pathways and may stimulate glioma-infiltrating microglia to express IL-10 (another T_H2 cytokine). Thus, although human glioma cells appear potentially sensitive to anti-tumor cell-mediated immunity, they are very efficient in preventing such responses from being generated.

The data from this study provides insight that may help design effective immunogene therapy strategies for human gliomas. Transferring genes encoding class II MHC and/or T cell costimulatory molecules such as B7-2 may augment glioma antigen presenting capacity. Transferring proinflammatory cytokine genes (GM-CSF, IL-12) should augment antigen presenting capacity and T_H1 responses, respectively. Finally, inhibiting immunoinhibitory cytokine (PGE2, TGF-β2) and/or T_H2 cytokine (IL-6) secretion, possibly by antisense strategies, may help stimulate activated anti-glioma cytotoxic T cells. Several of these strategies have been reported in preclinical studies involving rodent gliomas or gliosarcomas (13, 40, 52, 53). In our laboratory, we recently reported eradication of pre-established wild type tumor after vaccination with irradiated human glioma cells transduced with GM-CSF and B7-2 in an allogeneic human lymphocyte - severe combined immunodeficient (SCID) mouse model (45).

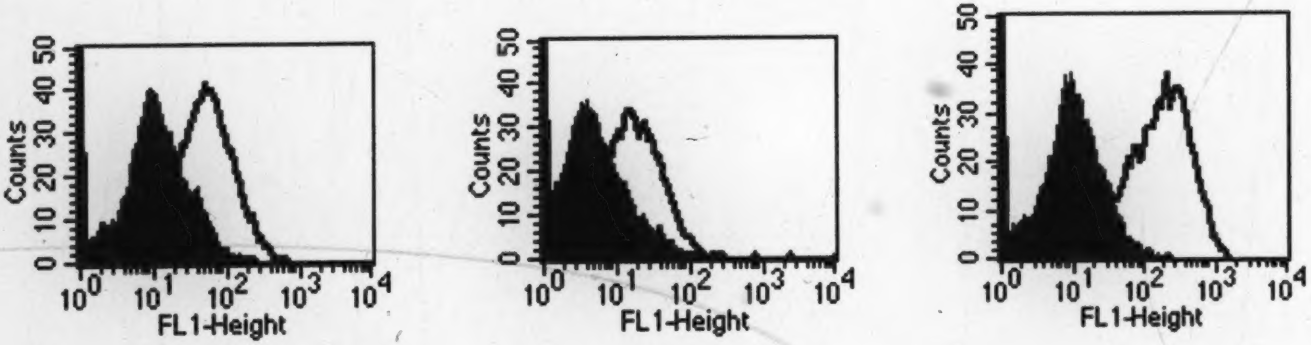
In summary, cultured human glioma cells (primarily early passage cultures) express significant amounts of class I MHC, Fas, IL-6, TGF-β2, and PGE2 but not class II MHC, B7-2, IL-10, IL-12, or GM-CSF. Although *in vivo* correlation is required, these results suggest that human glioma cells are potentially sensitive to anti-tumor T cell-mediated cytotoxicity. However, anti-inflammatory cytokine expression (IL-6, TGF-β2, PGE2) and lack of class II MHC molecules and T cell costimulators likely inhibit T cell activation *in vivo*. Considerably higher IL-6 levels were produced than TGF-β2 or PGE2, suggesting that IL-6 may be a more important cytokine in glioma immunobiology than previously appreciated. Conflicting results from previous immunogene therapy studies utilizing different animal tumor models suggested that an improved understanding of human glioma immunobiology was required to help design effective immunogene therapy strategies for human gliomas. We conclude that transferring immunostimulatory genes such as class II MHC, B7-2, GM-CSF, and IL-12 to human glioma cells or suppressing immunoinhibitory genes such as PGE2, TGF-β2, or IL-6 should promote cytotoxic T cell responses that are effective in eradicating wild type malignant glial cells.

Table 3-1. Summary of data from cultured glioma cells. For class I and II MHC, B7-2, and Fas, "Yes" represents positive staining by flow cytometry while "No" represents negative staining. For IL-6, IL-10, IL-12, GM-CSF, TGF-B2, and PGE2, values given are means from samples tested in triplicate and are listed as ng / 10^6 cells / 24 hours. N.D. = not done, MHC = major histocompatibility complex, IL-6 = interleukin-6, IL-10 = interleukin-10, IL-12 = interleukin-12, GM-CSF = granulocyte-macrophage colony-stimulating factor, TGF-B2 = transforming growth factor beta-2, and PGE2 = prostaglandin E_2.

Table 3-1

Code	Age	Sex	Pathology	Class I MHC	Class II MHC	B7-2	Fas	IL-6	IL-10	IL-12	GM-CSF	TGF-B2	PGE2
D54MG	N/A	N/A	Glioma	Yes	No	No	Yes	0.000	0.000	0.000	0.000	2.203	0.734
U251	N/A	N/A	Glioma	Yes	No	No	Yes	31.716	0.000	0.000	0.000	9.421	0.243
Ed77	39	F	Glioblastoma	Yes	No	No	Yes	N.D.	N.D.	0.000	1.550	N.D.	N.D.
Ed79	40	F	Glioblastoma	N.D.	N.D.	N.D.	N.D.	94.875	0.000	N.D.	N.D.	7.904	5.176
Ed84	22	F	Glioblastoma	N.D.	N.D.	N.D.	N.D.	16.247	0.000	0.000	N.D.	0.000	8.990
Ed85	52	M	Glioblastoma	N.D.	N.D.	N.D.	N.D.	11.117	0.000	0.000	N.D.	0.000	5.743
Ed93	61	M	Astrocytoma	Yes	No	No	Yes	97.162	0.000	0.000	0.200	71.038	47.506
Ed98	44	M	Glioblastoma	Yes	No	No	Yes	N.D.	N.D.	0.000	0.000	N.D.	N.D.
Ed99	56	F	Glioblastoma	Yes	No	No	Yes	4.794	0.000	0.000	0.000	7.737	2.687
Ed100	23	F	Glioblastoma	N.D.	N.D.	N.D.	N.D.	4.318	0.000	0.000	N.D.	0.000	4.749
Ed104	44	M	Glioblastoma	N.D.	N.D.	N.D.	N.D.	36.557	0.000	0.000	N.D.	0.000	55.006
Ed105	44	F	Oligodendroglioma	N.D.	N.D.	N.D.	N.D.	0.000	1.803	0.000	N.D.	0.000	62.370
Ed106	45	M	Glioblastoma	N.D.	N.D.	N.D.	N.D.	14.690	0.000	0.000	N.D.	0.000	17.535
Ed109	69	M	Glioblastoma	Yes	No	No	Yes	N.D.	N.D.	0.000	0.318	N.D.	N.D.
Ed110	46	M	Glioblastoma	N.D.	N.D.	N.D.	N.D.	51.314	0.000	N.D.	N.D.	0.000	30.769
Ed111	41	M	Glioblastoma	Yes	No	No	Yes	4.215	0.000	0.000	0.660	0.968	1.990
Ed113	60	F	Glioblastoma	Yes	No	No	Yes	7.820	0.966	0.000	0.000	3.856	14.794
Ed114	43	M	Glioblastoma	Yes	No	No	Yes	173.104	0.000	0.000	0.000	6.664	54.043
Ed116	25	M	Astrocytoma	Yes	No	No	Yes	2.554	0.000	0.000	0.310	4.059	1.778
Ed117	69	F	Glioblastoma	Yes	No	No	Yes	146.491	0.000	0.000	0.007	11.890	6.831
Ed118	65	F	Glioblastoma	Yes	No	No	Yes	28.370	0.766	0.000	0.000	45.491	3.423
Ed119	24	F	Glioblastoma	N.D.	N.D.	N.D.	N.D.	12.087	0.000	0.000	N.D.	4.768	0.363
Ed120	31	M	Glioblastoma	Yes	No	No	Yes	27.726	0.107	0.000	0.000	6.295	0.372
Ed121	41	M	Glioblastoma	N.D.	N.D.	N.D.	N.D.	2.125	0.000	0.000	N.D.	0.000	2.650
Ed147	57	M	Glioblastoma	Yes	Yes	No	Yes	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Ed149	45	M	Glioblastoma	Yes	No	No	Yes	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Figure 3-1. Class I MHC Expression



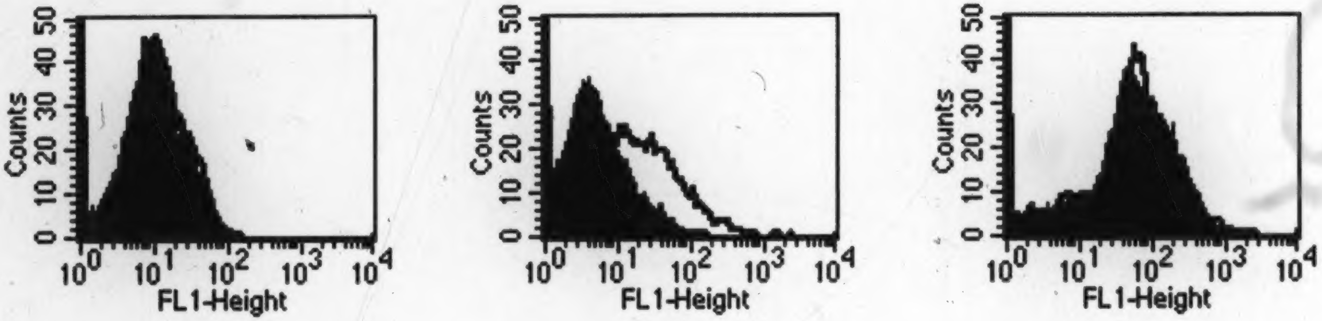
A. D54MG

B. Ed147.BT

C. Ed149.BT

Figure 3-1. Representative flow cytometry histograms showing class I MHC expression for: a) D54MG, b) Ed147.BT, and c) Ed149.BT. Shaded histograms represent background fluorescence with an isotype-matched control antibody. Unshaded histograms are fluorescence with anti-class I MHC antibody.

Figure 3-2. Class II MHC Expression



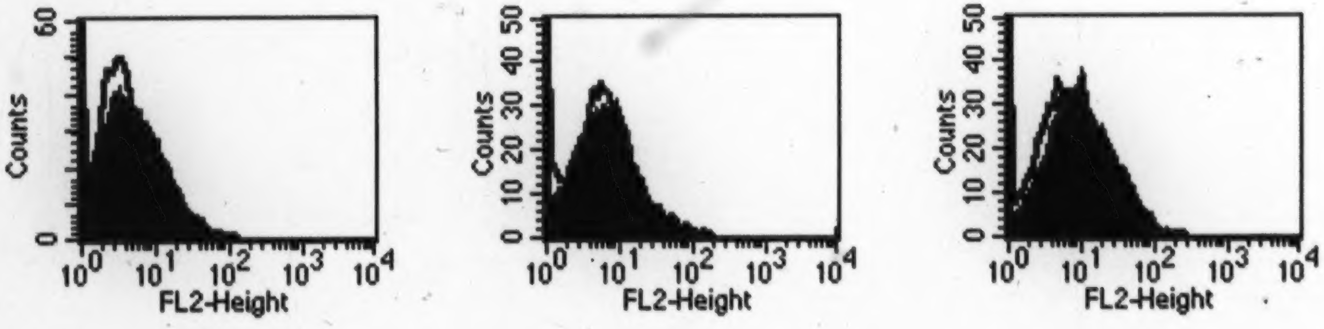
A. D54MG

B. Ed147.BT

C. Ed149.BT

Figure 3-2. Representative flow cytometry histograms showing class II MHC expression for: a) D54MG, b) Ed147.BT, and c) Ed149.BT. Shaded histograms represent background fluorescence with an isotype-matched control antibody. Unshaded histograms are fluorescence with anti-class II MHC antibody.

Figure 3-3. B7-2 Expression



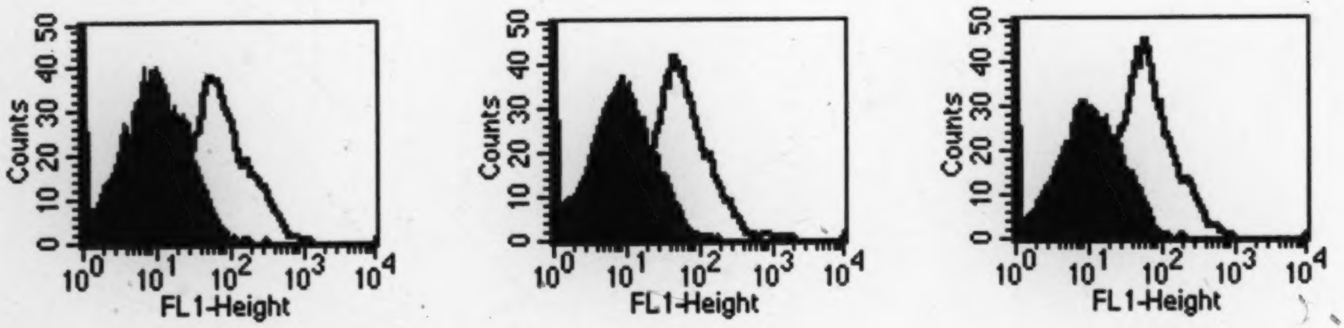
A. D54MG

B. Ed147.BT

C. Ed149.BT

Figure 3-3. Representative flow cytometry histograms showing B7-2 expression for: a) D54MG, b) Ed147.BT, and c) Ed149.BT. Shaded histograms represent background fluorescence with an isotype-matched control antibody. Unshaded histograms are fluorescence with anti-B7-2 antibody.

Figure 3-4. Fas Expression



A. D54MG **B. Ed147.BT** **C. Ed149.BT**

Figure 3-4. Representative flow cytometry histograms showing Fas expression for: a) D54MG, b) Ed147.BT, and c) Ed149.BT. Shaded histograms represent background fluorescence with an isotype-matched control antibody. Unshaded histograms are fluorescence with anti-Fas antibody.

Figure 3-5.

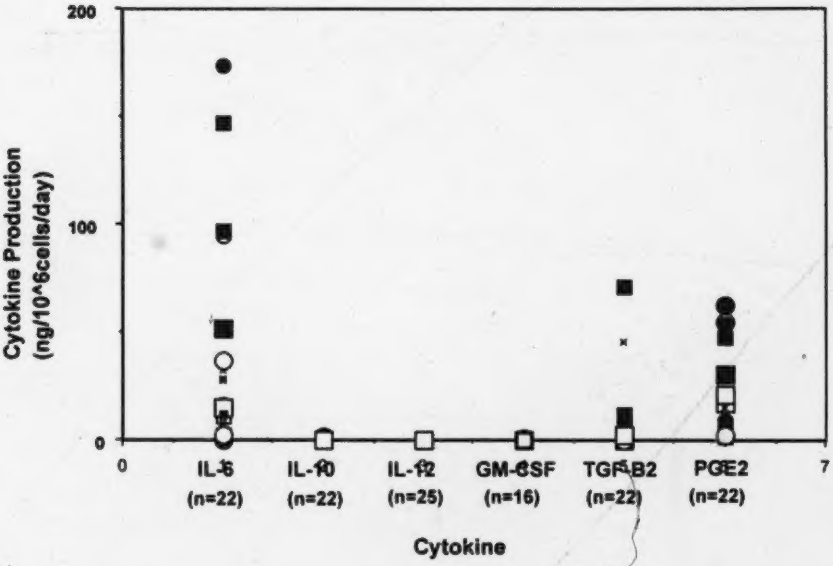


Figure 3-5. Cultured Human Glioma Cytokine Expression

Scatter plot showing cytokine expression levels for various human glioma cultures. Values shown are means from individual human glioma cell cultures tested in triplicate.

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Chapter 4

Human Gliomas Express Fas but not Fas-Ligand

Introduction

Fas/Apo1 (CD95) and Fas-ligand (FasL) are members of the tumor necrosis factor receptor (TNFR) and tumor necrosis factor (TNF) superfamilies (respectively) (11, 16, 20, 32). These families include TNFR-1, 2 / TNF, nerve growth factor receptor (NGFR) / nerve growth factor (NGF), CD27 / CD27 ligand, CD30 / CD30 ligand, CD40 / CD40 ligand, and TNF-related apoptosis inducing ligand (TRAIL) receptor / TRAIL (19). Both Fas and FasL exist as membrane-bound proteins, but FasL is often cleaved by a specific metalloproteinase to yield a soluble form (33, 34). FasL binding induces apoptosis (programmed cell death) in Fas^+ cells (19).

Fas is widely expressed on many human cell types while FasL is primarily expressed on natural killer cells and activated T cells (16, 32). The Fas / FasL system is responsible for a number of important physiologic functions including eliminating autoreactive lymphocytes (19). In addition, the Fas / FasL interaction is one of the main pathways for cytotoxic T cell-mediated killing and may be the most important pathway under physiologic conditions (2, 13, 27).

Because of the critical roles that Fas and FasL play in cell mediated immunity, it is possible that abnormal Fas / FasL expression by tumor cells may protect from immune surveillance. Tumor cells that down regulate Fas may be insensitive to cytotoxic T cell-mediated immunity (16, 17, 21, 22). $FasL^+$ tumor cells may also be resistant to immune attack as FasL expression implies insensitivity to Fas-mediated apoptosis (16, 17, 21, 22). In addition, FasL expression may induce apoptosis in naïve (Fas$^+$) T cells (3, 9, 10, 15, 31).

Our laboratory and others have demonstrated that most gliomas express Fas (1, 22, 24, 36). Exposing gliomas to FasL or agonistic anti-Fas antibodies appears to induce apoptosis (26, 36). Furthermore, cytotoxic T cells with anti-glioma activity exert their effects at least partly through Fas / FasL interactions (35). These findings support the hypothesis that gliomas are potentially sensitive to cell-mediated immunity and to treatment with FasL or anti-Fas antibodies.

In contrast, two recent reports have suggested that gliomas may express FasL based on rtPCR and immunohistochemical analysis (8, 28). Given gliomas' apparent Fas$^+$ status and sensitivity to Fas-mediated apoptosis, this is somewhat surprising. If confirmed, FasL expression would have important implications for many experimental glioma therapies. Our laboratory and others have recently reported marked glioma inhibition in pre-clinical models utilizing novel treatment strategies designed to stimulate anti-tumor cytotoxic T cells (5, 7, 12, 25, 29). Despite these results, one can predict that human gliomas would be significantly resistant to immunotherapy clinically if they are FasL$^+$ due to Fas-ligand's immunosuppressive effects. Similarly, despite promising pre-clinical results (26, 36, 37), "immunochemotherapy" via FasL or anti-Fas antibody administration may be ineffective if human gliomas are FasL$^+$ due to the implied insensitivity to Fas-mediated apoptosis.

Clearly, Fas and FasL expression patterns have important implications for glioma immunotherapy. However, contradictory results in the literature make it difficult to be certain concerning Fas and FasL expression in human gliomas. To help clarify these issues, we wished to examine Fas and FasL expression patterns in human glioma cells. Utilizing immunofluorescent staining with flow cytometric analysis and a novel indirect assay for FasL biologic activity, we tested seven human glioma cell cultures (three immortalized cell lines and four early passage cultures) for Fas and FasL expression.

Methods

Cell Culture

The U251 and U373 cell lines were obtained from the American Tissue Culture Collection (ATCC). Dr. D. Bigner (Duke University) graciously provided the D54MG cell line. The Ed147.BT, Ed149.BT, Ed168.BT, and Ed186.BT glioma cultures were derived from fresh operative glioblastoma specimens as previously described (23). The BW5147 cell line was provided by ATCC.

All cells were cultured at 37°C, 5% CO_2. U251 and D54MG were grown in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% fetal bovine serum, 200 uM L-glutamine, 100 mg/ml penicillin, and 100 IU/ml streptomycin. U373 was grown in similarly supplemented Eagle's Modified Eagle's Media (EMEM) Ed147.BT, Ed149.BT, Ed168.BT, and Ed186.BT were grown in DMEM/F12 media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 mg/ml penicillin, 100 IU/ml streptomycin, 100 uM sodium pyruvate, and 0.05 mM non-essential amino acids. BW5147 and BW-114.1 cells were grown in RPMI 1640 media supplemented with 10% fetal bovine serum, 200 uM L-glutamine, 100 mg/ml penicillin, and 100 IU/ml streptomycin. All media and additives were obtained from Gibco/BRL.

Fas / FasL Flow Cytometry

Glioma cells (adherent) were grown to ~ 70% confluence in T75 flasks, incubated for 30 minutes at 37°C in phosphate buffered saline (PBS; Gibco/BRL) with 0.04% EDTA, and harvested by pipetting and (when necessary) scraping. BW5147 and BW-114.1 cells (non-adherent) were harvested simply by pelleting. Cells were washed once in immunofluorescence buffer (IF buffer; 2% FBS, 0.02% sodium azide in PBS), then divided into aliquots of ~ 1 x 10^6 cells / 200ul and placed on ice in 1.5 ml Eppendorf tubes.

Staining for Fas and FasL proceeded similarly. One microliter of primary antibody (anti-human Fas, Pharmingen; or NOK-1/anti-human FasL, Pharmingen) or isotype-matched control antibody (X931/murine IgG1, Dako) was added to each 200ul aliquot. These were mixed by gentle agitation, then incubated on ice for 1 hour. Samples were washed twice with IF buffer, resuspended in 200 ul IF buffer with 10% normal goat serum, and incubated at room temperature for 10 minutes. Without further washing, 1 ul of secondary antibody (goat anti-mouse immunoglobulin-FITC; Dako) was added to each sample prior to incubating in the dark on ice for another hour. Samples were washed twice with IF buffer, then resuspended in 500 ul of 1% formaldehyde in PBS. These cells were then read immediately on the FL1 parameter on a Becton Dickinson flow cytometer using Cell Quest software. In addition to these assays, the human glioma cultures were also stained for FasL (as above) after 24 hour culture in media supplemented (1:1000) with FasL-specific matrix metalloproteinase inhibitor (Pharmingen) to see if this increased observable membrane-bound FasL expression.

Developing BW-114.1 (FasL-Sensitive Cell Line)

Because gliomas might be resistant to FasL-mediated killing even though they express Fas, we chose to develop a non-glioma cell line (BW-114.1) that was highly FasL-sensitive as an indicator cell line for FasL activity. BW-114.1 was developed as previously described (C. Hancock-Friesen, MSc Thesis, 1996). Briefly, the human Fas cDNA was kindly supplied by Dr. L.-J. Chang (Dept. of Medical Microbiology and Immunology, University of Alberta). This was amplified by reverse transcriptase polymerase chain reaction (rtPCR) and ligated into an eukaryotic expression vector (pRSaHFasR). The murine lymphoma cell line BW5147 was transfected with pRSaSD7 along with eukaryotic expression vectors encoding hypoxanthine phosphoribosyl transferase (pRSaHPRT) and dihydrofolate reductase (pRSaDHFR) via electroporation. A purified transfected population was selected by supplementing the growth media (RPMI 1640) with 100 uM hypoxanthine and 1 ug/ml azaserine. The resulting cell line was named

BW-114.1. Fas expression by BW-114.1 but not by wild type BW5147 was confirmed by flow cytometry as above.

Source of recombinant FasL

Recombinant FasL was prepared as previously described (C. Hancock-Friesen, MSc Thesis, 1996). Briefly, human FasL cDNA was amplified from total RNA from PHA-stimulated Jurkat cells by rtPCR and ligated into an eukaryotic expression vector (pRSaHFasL137). COS cells at 70% confluence were transiently transfected with pRSaHFasL137 or pRSaSD7 (empty vector controls) by calcium phosphate precipitation. Fresh media was added and cells were incubated for a further 48 hours. This media was harvested, centrifuged at 1000 rpm x 10 minutes, and frozen in 1 ml aliquots at -70°C for future use.

MTT Assay for Secreted FasL Bioactivity

1 x 10^5 BW-114.1 cells in RPMI 1640 were placed in each well of a 96 conical well plate (Falcon). Human glioma-conditioned media (0 ul, 1 ul, 10 ul, 40 ul, or 100 ul per well) was added in quadruplicate. The final volume was adjusted to 200 ul / well with RPMI 1640. Wells with conditioned media (0 ul, 0.5 ul, 1 ul, or 5 ul) from COS cells transduced with FasL (pRSaHFasL137) or

empty vector (pRSaSD7) + / - 1 ul of anti-FasL antibody (NOK-1, Pharmingen) were used as controls.

Cells were incubated for 24 hours at 37°C, 5% CO_2. Following this, the MTT assay was performed as described previously (18). MTT reagent (50 ul of 5 mg/ml) was added to each well and the plate was incubated for a further 6 hours at 37°C, 5% CO_2. The resulting crystals were pelleted by centrifuging the plate for 15 minutes at 1000 rpm. The supernatant was removed by aspiration, and 100 ul of isopropanol was added to each well. After 30 minutes incubation at room temperature, the OD490 of each well was measured with a spectrophotometer.

Assay for membrane-bound FasL bioactivity

Human glioma cells were plated in duplicate in 24 well plates at 0, 1.5×10^4, 7.5×10^4, 1.5×10^5, or 3.0×10^5 cells / well. These were incubated overnight at 37°C, 5% CO_2 in 2 ml/well of appropriate growth media (DMEM, DMEM/F12, or EMEM). The following day, the supernatant was removed by aspiration and either 5×10^5 BW-114.1 (Fas^+) or 5×10^5 BW5147 (wild type) cells were added to each well in 500 ul RPMI 1640 to yield effector to target cell ratios of 0:1, 0.03:1, 0.15:1, 0.3:1, and 0.6:1. Cells were incubated for 30 minutes at 37°C, 5% CO_2. BW-114.1 or BW5147 cells (in suspension) were harvested for apoptosis staining by transferring the media from each well to 1.5 ml Eppendorf tubes, leaving the adherent glioma cells behind. After being washed once with PBS, BW5147 /

BW-114.1 cells were immediately stained with anti-annexin V-FITC and propidium iodide (PI) using a commercially available kit (R + D Systems, Gibco-BRL) as per the manufacturer's instructions. Cells were read on the FL1 and FL2 parameters using a Becton Dickison flow cytometer and Cell Quest software. Cells negative for both Annexin V and PI were interpreted as "viable." AnnexinV (+) / PI (-) cells were interpreted as "early apoptotic." Annexin V (+) / PI (+) cells were interpreted as "late apoptotic" and "necrotic."

Results

All (7/7) human glioma cultures stained positively for Fas by flow cytometry (**Figure 4-1**). None (0/7) stained positively for FasL (**Figure 4-2**). Culturing the gliomas for 24 hours in the presence of FasL-specific matrix metalloproteinase inhibitor to reduce cleavage to the soluble form did not increase observable FasL expression by flow cytometry (data not shown).

Co-transfecting BW5147 cells with pRSaHFasR, pRSaHPRT, and pRSaDHFR and selecting with hypoxanthine and azaserine resulted in a cell line (BW-114.1) phenotypically indistinguishable from BW5147 except regarding human Fas expression. BW5147 cells were Fas$^-$ while BW-114.1 cells were Fas$^+$ (**Figure 4-3**). By MTT assay, exposing BW-114.1 cells to increasing concentrations of conditioned media from human FasL-transfected COS cells resulted in progressive growth inhibition. These inhibitory effects could be blocked by the addition of NOK-1 (anti-FasL blocking antibody) and were not seen after the addition of conditioned media from pRSaSD7 (empty vector)-transfected COS cells (**Figure 4-4**). These results confirmed BW-114.1's sensitivity to human FasL. Exposing BW-114.1 to conditioned media from the seven human glioma cultures did not result in significant growth inhibition (even at concentrations as high as 50%), strongly indicating that the gliomas do not secrete FasL (**Figure 4-5**).

Ideally, the annexin V-FITC (AnV) / propidium iodide (PI) flow cytometry assay should differentiate between viable cells (AnV$^-$ / PI$^-$), apoptotic cells (AnV$^+$ / PI$^-$), and necrotic cells (AnV$^+$ / PI$^+$). However, this is very time dependent. As apoptosis progresses, cell membranes breakdown. This allows propidium iodide to enter the cell and bind to nucleic acids. Thus while very early apoptotic cells stain AnV$^+$ / PI$^-$, later apoptotic cells become AnV$^+$ / PI$^+$. In practice, although glioma co-culture appeared to induce an increase in AnV$^+$ / PI$^-$ (apoptotic) BW5147 / BW-114.1 cells, we found it difficult to harvest the cells and complete the staining with sufficient speed to avoid a concomitant increase in AnV$^+$ / PI$^+$ cells. Therefore, the combined total of AnV$^+$ / PI$^-$ (early apoptotic) and AnV$^+$ / PI$^+$ (late apoptotic and necrotic) BW5147 / BW-114.1 cells was taken as a measure of apoptotic cell death in response to glioma co-culture. A representative series of AnV / PI dot plots for BW5147 and BW-114.1 cells cultured with and without Ed168.BT cells is shown in **Figure 4-6**.

BW-114.1 cells underwent increased apoptotic cell death in a dose dependent fashion after co-culture with all the gliomas except D54MG (**Figure 4-7B**). This was greatest for Ed186.BT and least for U251. Initially, we felt that this might represent membrane-bound FasL activity on the gliomas, despite the fact that we could not demonstrate membrane bound FasL by flow cytometry (**Figure 4-2**). However, subsequent control experiments with wild type (Fas^{+}) BW5147 cells resulted in nearly identical increases in apoptotic cell death (**Figure 4-7A**). This strongly suggests that the apoptotic cell death induced in BW5147 and BW-114.1 cells by human glioma co-culture is not mediated by Fas / FasL interactions.

Conclusions

All the human glioma cultures we tested (7/7) showed evidence for Fas expression by flow cytometry (**Figure 4-1**). This is consistent with published results from our laboratory and others suggesting that most gliomas are Fas^{+} (1, 22, 24, 36). Fas expression is highly variable in different tumor types (16, 17, 21, 22, 38). Interestingly, in the reports by Hahne *et al.* and Strand *et al.* in which FasL expression by human tumors was originally reported, tumors and tumor cell lines expressing FasL appeared to have down-regulated Fas expression and were insensitive to FasL-mediated apoptosis (10, 31). While not all Fas^{+} tumors are necessarily sensitive to FasL (22), these early reports suggest that most FasL^{+} tumors down-regulate Fas to avoid inducing apoptosis in themselves.

We were not able to demonstrate convincing evidence that any of the seven human glioma cultures we tested expressed FasL. None (0/7) were positive for membrane-bound FasL by flow cytometry (**Figure 4-2**). None (0/7) produced conditioned media that killed the FasL-sensitive cell line BW-114.1 **(Figure 4-5)**, suggesting that none secreted FasL. Although 6/7 glioma cultures induced apoptosis in Fas$^+$ BW-114.1 cells after short term co-culture, the same cultures induced apoptosis in Fas$^-$ BW5147 cells, indicating that the apoptosis was not mediated by membrane-bound FasL **(Figure 4-7)**.

The fact that the human glioma cultures in our study do not secrete FasL is consistent with previous reports but their lack of membrane-bound FasL expression is not (8, 28). The reasons for this discrepancy are not entirely clear. It has been reported that some common commercially available anti-human FasL antibodies (C-20, Santa Cruz Biotechnology; mAb33, Transduction Laboratories) may yield false positive results, depending on the assays used (6, 30). C-20 appears reliable for immunoblotting but not for flow cytometry; mAb33 is unreliable for either assay. NOK-1 (the anti-human FasL antibody used in the present study) appears to be reliable (6, 14, 30). In their reports of FasL expression by gliomas, neither Saas *et al.* or Gratas *et al.* used commonly available commercial antibodies to demonstrate FasL expression directly (8, 28). However, Saas *et al.* showed that NOK-1 could block glioma-derived FasL activity (28). This suggests that discrepancies between our results and previously published reports cannot be ascribed simply to unreliable antibody staining.

These discrepancies may reflect the fact that different glioma cell cultures were tested and may be due to variations from cell line to cell line. However, the fact that all of our cell cultures were negative for FasL suggests that pure chance variation does not account for these differences. Alternatively, our cultures may be derived in such a way that FasL $^-$ populations are specifically selected and supported. Gratas *et al.* noted that different regions within gliomas had different FasL expression patterns when immunohistochemical studies of paraffin sections were performed (8). It is possible that our techniques for generating and propagating human glioma cultures (23) coincidentally result in FasL $^-$ cell populations. However, this would not explain the FasL $^-$ status of immortalized human glioma cell lines such as D54MG, U251, and U373 that were not prepared in our laboratory unless the methods used to prepared these cell lines (4) also specifically promoted FasL$^-$ cell growth. Furthermore, given that our early passage cultures (Ed168.BT, Ed186.BT) were also FasL $^-$, it seems unlikely that FasL absence is simply the result of prolonged *in vitro* culture.

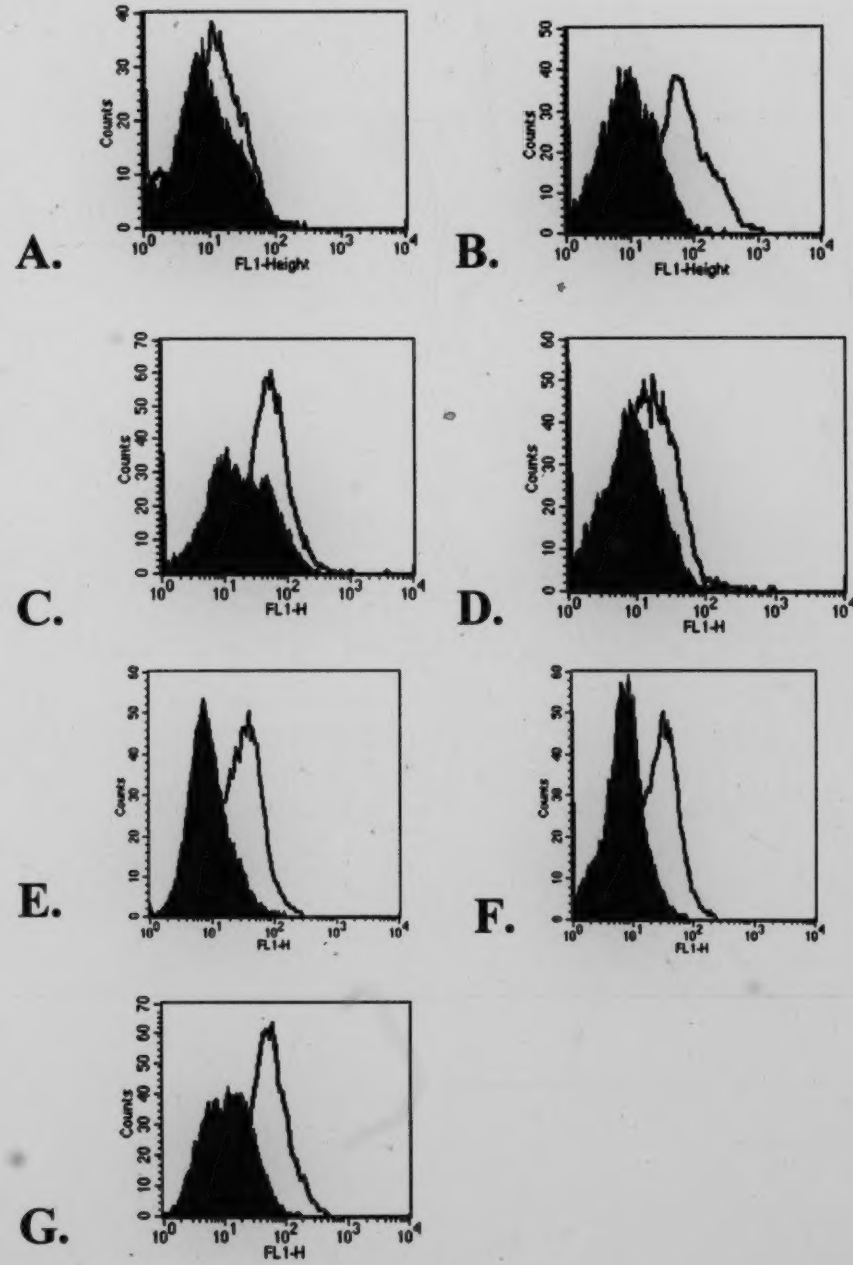
Despite their apparent lack of secreted or membrane-bound FasL, our results indicate that human gliomas may express another as yet unidentified factor that may have similar immunosuppressive effects to those expected for FasL. Short-term human glioma co-culture rapidly induces cell death in a murine lymphoma cell line (BW5147) in a non-Fas/Fas-ligand-mediated fashion (**Figure 4-7B**). This is an unexpected and surprising finding. An increase in AnV $^+$ / PI $^{+/-}$ BW5147 cells with glioma co-culture suggests that this was apoptotic cell death. If this work can be replicated in other lymphoid cell lines and (more importantly) in human peripheral blood mononuclear cells, it would suggest that gliomas possess another as yet uncharacterized immunosuppressive pathway

In summary, our results confirm that most human gliomas express Fas. FasL expression was absent in all the glioma cell lines we tested, although others have reported FasL expression by some human gliomas. Fas expression in the absence of Fas-ligand suggests that gliomas are likely to be sensitive to Fas-mediated apoptosis, the major mechanism of T cell-mediated killing. Furthermore, absence of FasL in all our human glioma samples suggests that this is not a major mediator of glioma-derived immunosuppression. These results are promising for proponents of both immunotherapy and Fas-ligand-mediated therapy for gliomas. However, human gliomas are able to rapidly induce apoptosis in a co-cultured lymphoid cell line (BW5147) in a non-Fas / FasL-mediated fashion. This suggests that they may possess other pathways to induce apoptosis in infiltrating lymphocytes that have not yet been well characterized.

Figure 4-1. Human Glioma Cell Line Fas Expression

Fas expression by flow cytometry in A) Ed147.BT, B) Ed149.BT, C) Ed168.BT, D) Ed186.BT, E) D54MG, F) U251, and G) U373. Shaded histograms represent background fluorescence when stained with an isotype-matched control antibody. Dark single line histograms represent fluorescence when stained with a monoclonal antibody directed at human Fas (CD95). A shift to the right compared to the control antibody represents positive staining.

Figure 4-1.


A.
Counts
0
10
20
30
40
FL1-Height
10^0
10^1
10^2
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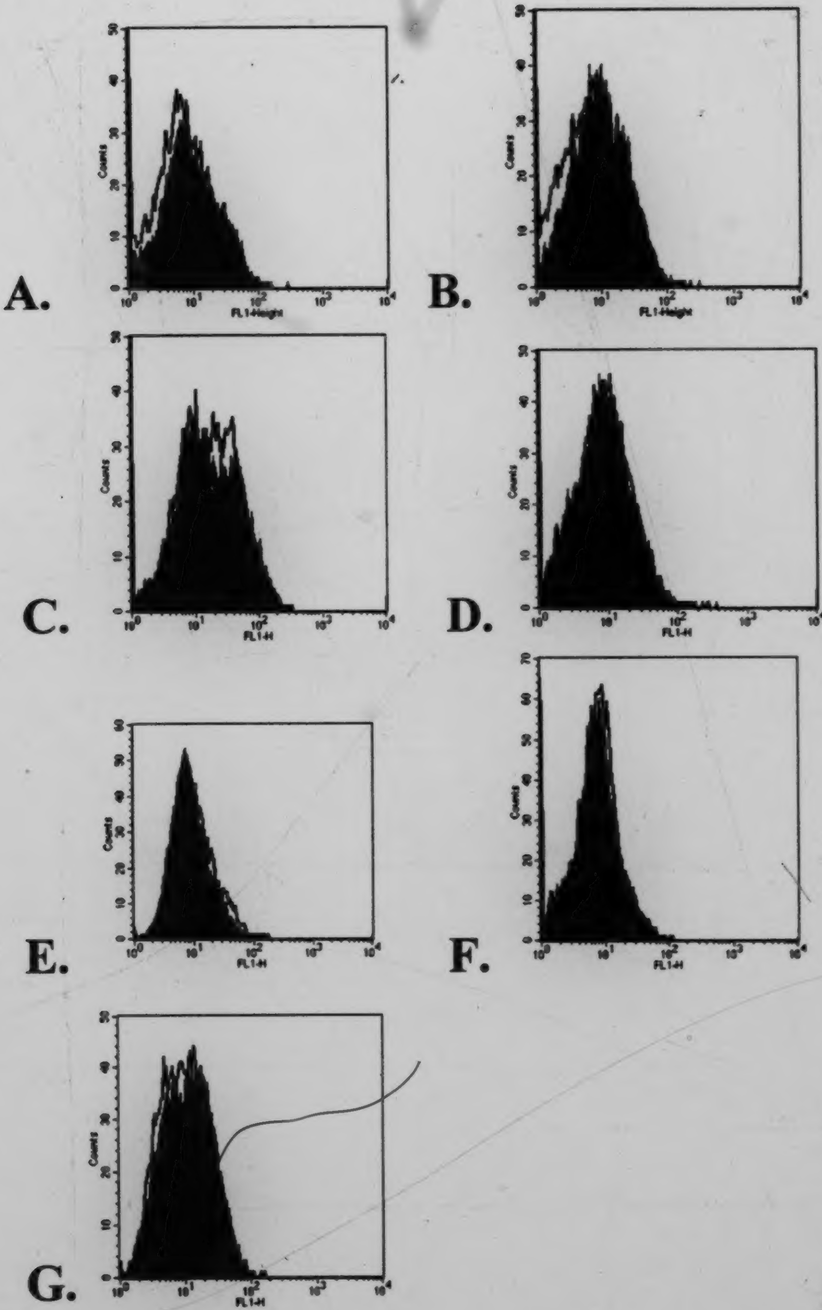
10^4
B.
Counts
0
10
20
30
40
50
FL1-Height
C.
Counts
0
10
20
30
40
50
60
70
FL1-H
D.
Counts
FL1-H
E.
Counts
FL1-H
F.
Counts
FL1-H
G.
Counts
FL1-H

Figure 4-2. Lack of FasL Expression in Human Glioma Cell Lines

Lack of FasL expression by flow cytometry in A) Ed147.BT, B) Ed149.BT, C) Ed168.BT, D) Ed186.BT, E) D54MG, F) U251, and G) U373. Shaded histograms represent background fluorescence when stained with an isotype-matched control antibody. Dark single line histograms represent fluorescence when stained with a monoclonal antibody directed at human FasL. A shift to the right compared to the control antibody would represent positive staining.


A.
B.
C.
D.
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Counts
FL1-Height
FL1-H
10^0
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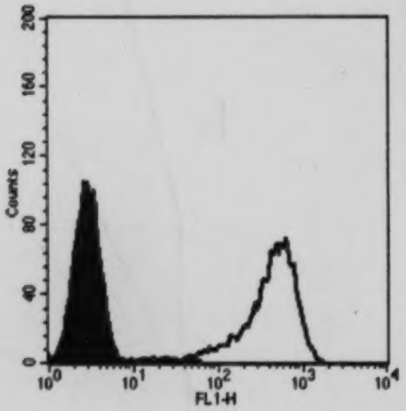
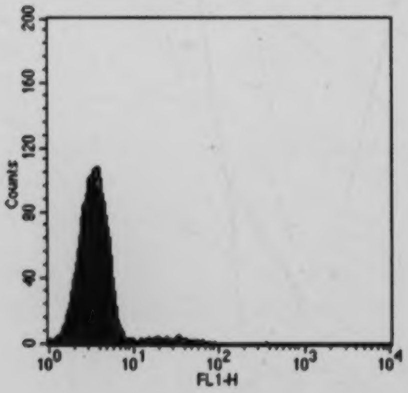
10^2
10^3
10^4

Figure 4-3.



A. BW-114.1



B. BW5147

Figure 4-3. Fas Expression in BW-114.1 Cells but not BW5147 Cells

Flow cytometry histograms demonstrating Fas expression by BW-114.1 cells but not BW5147 cells. Solid gray histograms represent background fluorescence with an isotype-matched control antibody. Dark single line histograms represent fluorescence with a Fas-specific antibody.

Figure 4-4.

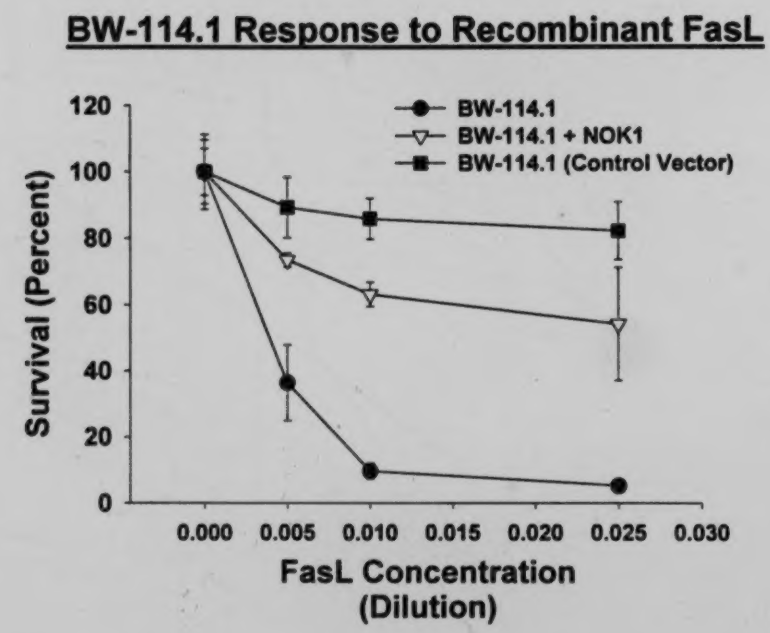
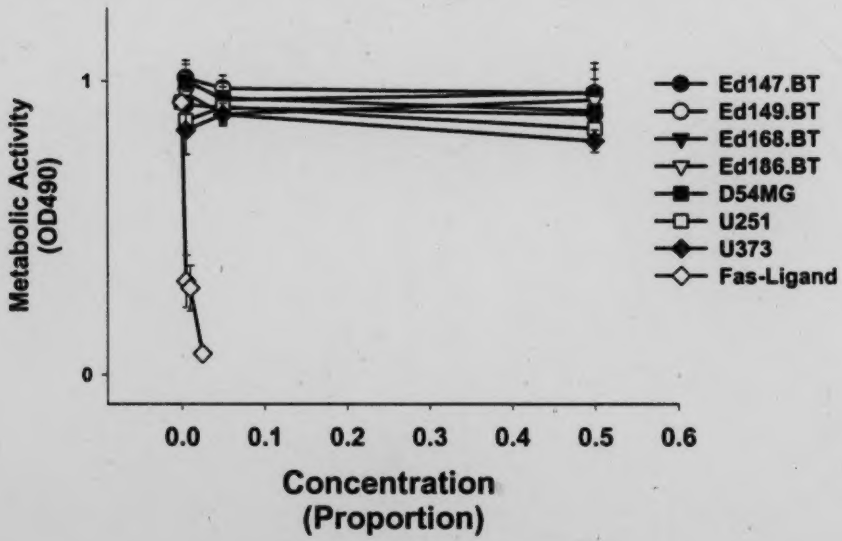


Figure 4-4. BW-114.1 Fas-Ligand Sensitivity

BW-114.1 cell killing by MTT assay after exposure to recombinant Fas-ligand. Significant killing (reduced metabolic activity) is seen with exposure to increasing concentrations of FasL-containing culture media (from pRSaFasL137-transfected COS cells). This is not seen with exposure to control media from cells transfected with empty vector (pRSaSD7). FasL-mediated killing is blocked by exposure to anti-human FasL antibody (NOK-1, Pharmigen). Metabolic activity shown as percentage of baseline (mean +/- standard deviation for experimsents performed in quadruplicate).

Figure 4-5.



BW-114.1 Response to Glioma Culture Supernatants

Figure 4-5. Lack Secreted Fas-Ligand in Human Glioma Supernatants

Metabolic activity after exposure to increasing concentrations of glioma-conditioned media dose not result in significant BW-114.1 cell killing. Metabolic activity (OD490) is represented as mean +/- standard deviation of experiments performed in quadruplicate. Controls exposed to media containing recombinant Fas-ligand show marked cell killing. This strongly suggests that glioma cells do not secrete Fas-ligand *in vitro*.

Figure 4-6.

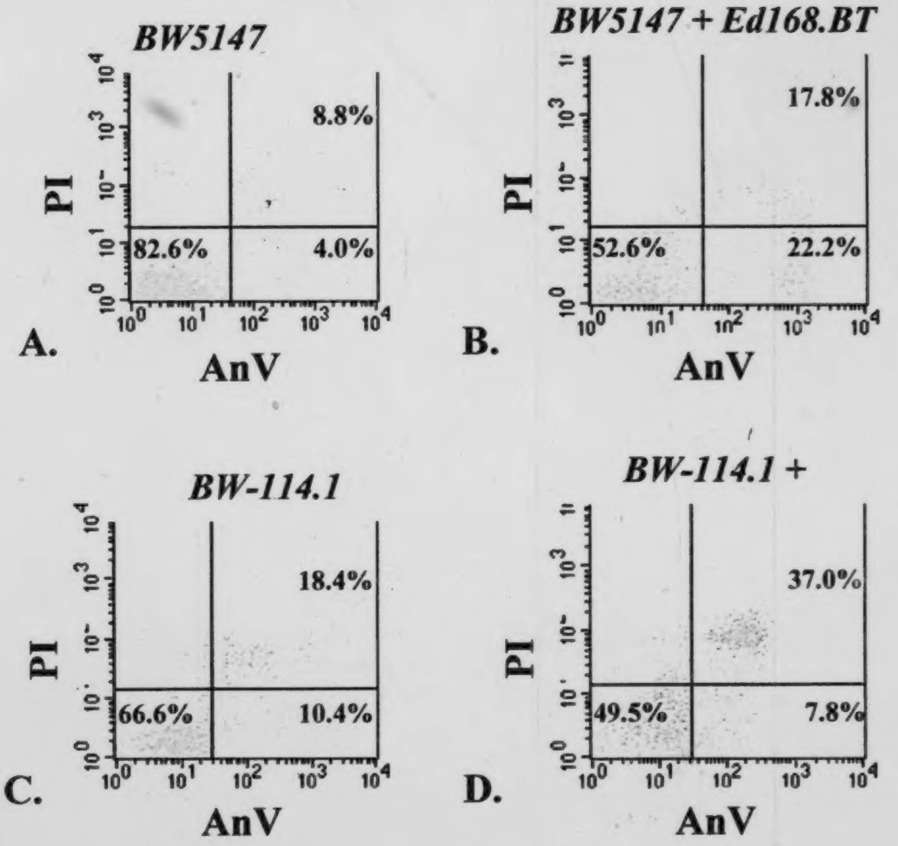


Figure 4-6. Annexin V-FITC / Propidium Iodide Dot Plots

Representative dot plots of Annexin V-FITC (AnV, x-axis) *vs.* propidium iodide (PI; y-axis) for BW5147 and BW-114.1 cells. AnV$^-$ / PI$^-$ cells are viable. AnV$^+$ / PI$^-$ cells are in early apoptosis. AnV$^+$ / PI$^+$ are in late apoptosis or necrosis. A) BW5147, B) BW5147 + 30 minute Ed168.BT co-culture (effector to target ratio = 0.6:1), C) BW-114.1, D) BW-114.1 + 30 minute Ed168.BT co-culture (effector to target ratio = 0.6:1).

Figure 4-7. Non-Fas-Mediated Killing Induced in Murine Lymphoma by Short Term Human Glioma Co-Culture.

A) Coculture with human gliomas for 30 minutes increases apoptotic/necrotic BW5147 cells (Fas$^-$) in a dose dependent fasion for all cultures tested except D54MG. For all cultures, this effect is as large or larger than that seen with BW-114.1.

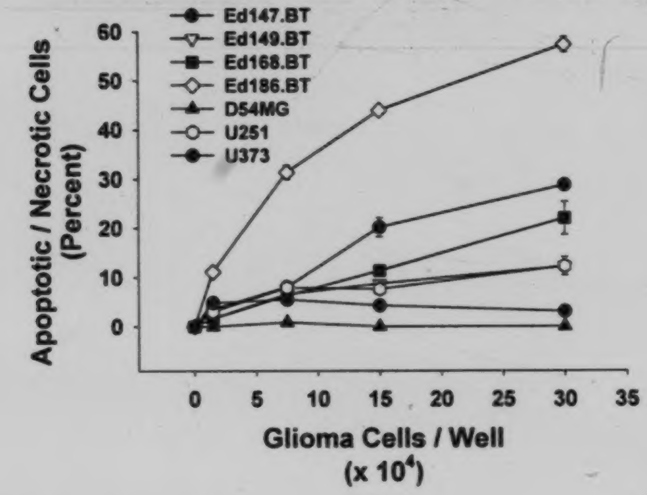
B) Coculture with human gliomas for 30 minutes increases apoptotic/necrotic BW-114.1 cells (Fas$^+$) in a dose dependent fasion for all cultures tested except D54MG.

Means from experiments performed in duplicate +/- standard deviation are shown. Background apoptosis / necrosis has been subtracted.

Figure 4-7.

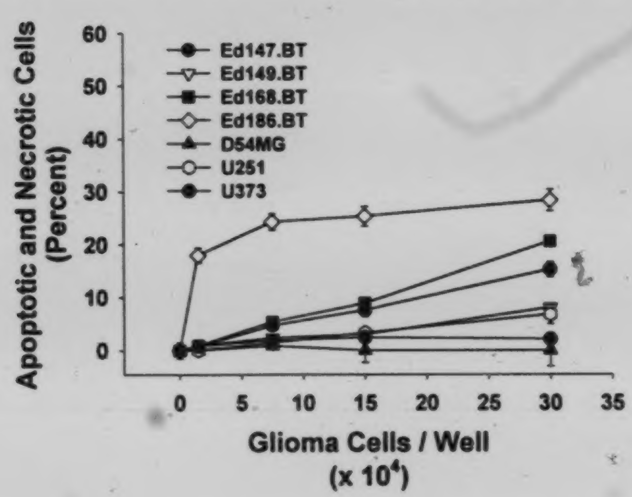
A.

BW5147 Response to Human Glioma Co-Culture



B.

BW-114.1 Response to Human Glioma Coincubation



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Chapter 5

Human Brain Tumor Cell Culture Characterization Following Immunostimulatory Gene Transfer

Introduction

Recent advances in immunology and molecular biology have led to the development of cancer immunogene therapy (1, 4, 13). Although there are many potential forms of immunogene therapy, it usually involves vaccination with autologous tumor cells that have been genetically altered to increase their immunogenicity. Prior to administration to patients, these genetically altered cells are irradiated to prevent further cell division.

Many different ways of genetically altering these tumor cells have been proposed. These involve either transferring pro-inflammatory genes or inhibiting immunosuppressive gene expression. Commonly transferred pro-inflammatory genes include granulocyte-macrophage colony-stimulating factor (GM-CSF) (4), interleukin-2 (IL-2) (7), interleukin-12 (IL-12) (27), and T cell costimulatory molecules (B7-1, B7-2) (10). Transforming growth factor β's (particularly TGF-β2) have been targeted as a commonly expressed immunosuppressive factors in many tumors (5).

Glioma immunobiology suggests that these tumors may be particularly well suited to immunogene therapy. Human gliomas appear to express class I MHC and Fas (CD95), suggesting that they may be recognized and killed (respectively) by cytotoxic ($CD8^+$) T cells (21, 28). However, they express a number of immunosuppressive factors (TGF-β2, prostaglandin E_2, interleukin-10, and, possibly, Fas-ligand) that inhibit T cell activation (9, 11, 12, 15, 26). Taken together, this suggests that human glioma cells are potentially sensitive to cell-mediated immunity but normally suppress activation of immune responses. Immunogene therapy (either by transferring potent pro-inflammatory genes to glioma cells or inhibiting expression of immunosuppressive factors) may be one way to overcome this immunosuppression and generate immune responses that are effective against wild type tumor cells.

Our laboratory (22) and others have recently reported impressive preclinical results utilizing immunogene therapy to treat brain tumors (5, 7, 8, 16, 24, 25, 29). Based on these results, we are considering clinical applications of this treatment. However, prior to embarking on clinical trials, several questions need to be addressed to help establish the safety and feasibility of vaccination with irradiated, genetically modified tumor cells. First, we wished to know whether our retroviral vectors were effective at transferring immunostimulatory genes to human brain tumor cells (in particular, early passage cultures derived from patients). As these cells would be irradiated prior to injection, we wished to know if irradiation affected the expression of the transferred genes. Since the purpose of irradiation is to prevent cell division (and, therefore, secondary tumor growth at vaccination sites) without killing the immunogenetically altered tumor cells, we wished to document irradiation's effects on their viability and clonogenicity. Finally, as clinical applications require large numbers of genetically altered cells, we wished to examine whether therapeutic gene transfer alters *in vitro* growth rates.

Methods

Cell Culture

D54MG is an immortalized human glioma cell line graciously provided by Dr. D. Bigner (2). It was cultured at 37°C, 5% CO_2 in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 I.U./ml penicillin, and 100 ug/ml streptomycin (media and supplements provided by Gibco/BRL).

Ed147.BT and Ed149.BT cell cultures were derived (respectively) from 52 year old and 49 year old males that both harbored glioblastoma multiforme tumors. These were processed using previously described techniques (20). Briefly, fresh surgical tumor specimen was minced into small (1 - 2 mm) pieces then subjected to partial enzyme digestion with pronase, DNase, and collagenase. The resulting slurry was filtered through a tissue culture sieve then centrifuged on a density gradient (Ficoll-Paque; Pharmacia) to remove contaminating mesenchymal cells such as fibroblasts. Cells were cultured at 37°C, 5% CO_2 in Dulbecco's modified Eagle's / F12 media (DMEM/F12) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 ug/ml streptomycin, 0.05 mM non-essential amino acids, and 100 uM sodium pyruvate (media and supplements provided by Gibco/BRL).

Ed141.MEL was derived from a 54 year old male harboring a malignant melanoma with secondary cerebral metastases. The technique used to establish melanoma cultures differed somewhat from that for gliomas. After mincing the specimen into 1 - 2 mm pieces, partial enzyme digestion was carried out with collagenase II and hyaluronidase. Cells were plated out directly (without density gradient centrifugation) and cultured at 37°C, 5% CO_2 in RPMI 1640 media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 I.U./ml penicillin, and 100 ug/ml streptomycin (media and supplements provided by Gibco/BRL).

Cloning B7-2, GM-CSF, and IL-12 Gene Transfer Vectors

cDNA's for B7-2, GM-CSF, IL12A, and IL12B were generated by reverse transcriptase polymerase chain reaction (rtPCR) using primers designed from previously published gene sequences available from the NCBI Gene Bank. These were ligated into pLSN, a replication-incompetent retroviral gene therapy vector derived from the Maloney leukemia virus (23). This yielded replication-incompetent retroviral gene therapy vectors encoding B7-2 (pLSNB70), GM-CSF (pLSNGM1), IL12A and IL12B (pLSNIL12), or B7-2 and GM-CSF (pLSNBG9). Bicistronic vectors that contained two genes under the control of a single promotor region (pLSNIL12, pLSNBG9) linked these genes with an internal ribosome entry site (IRES) derived from the encephalomyocarditis virus genome (Novagen). In addition to the therapeutic genes (all under the control of an LTR promotor), these vectors also contain a neomycin-resistance gene under the control of an SV40 promotor. This allows for neomycin-mediated cell selection after retroviral transduction.

Generation of Replication-Incompetent Retrovirus

Plasmid DNA for the gene therapy vectors described above was transfected into the retroviral packaging cell line PG13 (American Type Culture Collection) using calcium phosphate precipitation. This cell line expresses the retroviral packaging genes *gag*, *pol*, and *env* that are absent from the retroviral plasmid vectors. This allows packaging of infectious virus. However, these viruses still lack *gag*, *pol*, and *env* genes, rendering them replication-incompetent (18, 19). Culture supernatants containing virus were harvested 24 hours after plasmid DNA transfection and stored in 0.5 ml aliquots at -80°C for future use.

Retroviral Transduction and Cell Selection

Tumor cells (D54MG, Ed141.MEL, Ed147.BT, Ed149.BT) were grown to 70% confluence in T25 flasks. Virus (pLSNB70, pLSNGM1, pLSNIL12, or pLSNBG9) aliquots were thawed at 37°C. In the presence polybrene (4 μg/ml), cells were incubated with virus (brought to a final volume of 1.5 ml with appropriate fresh culture media) at 37°C, 5% CO_2 for 3 hours. In a separate flask, cells were incubated with the same volume of media and polybrene without virus as a control. After 3 hours, a further 3 ml of culture media was added to each flask and they were incubated overnight. Media was changed the next morning. Selective media containing 500 μg/ml of the neomycin analogue G418 was added. Media was changed every two to three days until complete selection had taken place (i.e. all cells in the control flasks were dead). After selection, transduced cells were cultured in growth media containing reduced concentrations of G418 (250 μg/ml) and allowed to grow to confluence.

B7-2 Expression Analysis

B7-2 expression was analyzed by flow cytometry. Wild type, pLSNB70-transduced, and pLSNBG9-transduced tumor cells grown to 70% confluence were harvested by incubating in 0.04% EDTA in PBS for 30 minutes at 37°C to detach them from their flasks. Irradiated cells (200 Gy) were harvested similarly after being cultured (5% CO_2, 37°C) overnight following irradiation. Samples were washed twice in immunofluorescence buffer (IF buffer; 2% fetal bovine serum with 0.02% sodium azide in PBS) and resuspended in aliquots of 1 x 10^6 cells / 200 ul IF buffer in 1.5 ml Eppendorf tubes. Samples were incubated for 1 hour on ice with either 1 ul of anti-B7-2-RPE antibody (Ancell) or 1 ul of isotype matched Ig-RPE (Pharmingen). After washing two times in IF buffer, cells were then resuspended in 500 ul of 1% formaldehyde in PBS and read immediately on the FL-2 parameter on a Becton-Dickison flow cytometer using CellQuest software.

GM-CSF and IL-12 Expression Analysis

Commercially available ELISA kits for GM-CSF and IL12 (R + D Systems, Gibco/BRL) were used to determine concentrations of these cytokines in 24 hour conditioned culture media from wild type, pLSNGM1, pLSNBG9, and pLSNIL12-transduced cultures (+/- 200 Gy). Cells were counted after harvesting media, enabling GM-CSF and IL-12 production to be converted to ng / 10^6 cells / day.

Viability Analysis

Wild type, pLSNB70, pLSNGM1, pLSNBG9, and pLSNIL12-transduced cultures were grown to 70% confluence in 6 well plates (Falcon) then irradiated (200 Gy). Unirradiated cells were used as controls. Wells were harvested in duplicate by trypsinization immediately following irradiation (day 0) and on days 1, 3, 5, and 10. Viability was assessed via trypan blue staining (viable cells exclude trypan blue; dead cells do not).

Clonogenicity Analysis

Wild type, pLSNB70, pLSNGM1, pLSNBG9, and pLSNIL12-transduced tumor cells (+/- 200 Gy) were plated out in quadruplicate in 6 well plates at 0, 250, 750, and 1000 cell/well. When colonies were visible to the naked eye on the unirradiated plates (~ 2 weeks), media was removed and methylene blue staining was employed to increase the visibility of the colonies. The number of colonies/well for each cell type (+/- irradiation) at each cell concentration was then counted.

Growth Curves

Growth curves were obtained for wild type, pLSNB70, pLSNGM1, pLSNBG9, and pLSNIL12-transduced tumor cells by plating 5 x 10^3 cells / well in 6 well plates. At 1 to 3 day intervals, cells from successive wells were harvested by trypsinization and counted using a hemocytometer using trypan blue staining. These experiments were performed in duplicate.

Results

Schematic plasmid maps for pLSNB70, pLSNGM1, pLSNBG9, and pLSNIL12 are shown in **Figure 5-1**. Our retroviral vectors were effective at transferring B7-2, GM-CSF, and IL-12 genes to human brain tumor cultures. B7-2 expression levels varied from 1 to 2 log increase in relative fluorescence by flow cytometry for all cell cultures transduced with either pLSNB70 or pLSNBG9 (**Figure 5-2, Table 5-1**). GM-CSF expression levels were high (9.795 - 46.701 ng/10^6 cells/day) for cells transduced with pLSNGM1 (**Table 5-1**). GM-CSF expression levels in cells transduced with pLSNBG9 and IL-12 expression levels in cells transduced with pLSNIL12 (1.241 - 7.794 and 1.302 - 2.104 ng/10^6 cells/day respectively) were somewhat lower. This likely represents reduced expression of genes downstream from the IRES site in our vectors. Therapeutic gene expression levels are summarized in **Table 5-1**.

Irradiation did not significantly affect gene expression. B7-2 expression by flow cytometry was not significantly altered 24 hours post-irradiation (200 Gy) (**Figure 5-2, Table 5-1**). Similarly, while there where minor variations in GM-CSF and IL-12 expression levels pre- and post-irradiation in individual cell cultures (**Table 5-1**), there was no significant difference in mean GM-CSF and IL-12 expression levels when the data from the different cell types was pooled (**Figure 5-3**).

Viability was initially high post-irradiation (94.20 +/- 8.46% on day 1). After this, cells began to die rapidly and only 28.13 +/- 4.64% were viable by day 10. Viability curves post-irradiation are shown in **Figure 5-4**. Unirradiated cells transduced with pLSNB70, pLSNGM1, pLSNBG9, and pLSNIL12 all remained viable over the same time period (data not shown). Irradiation with 200 Gy completely blocked all clonogenic potential for all the cell cultures tested suggesting that cell division was completely inhibited (**Figure 5-5**). Unirradiated cells transduced with pLSNB70, pLSNGM1, pLSNBG9, and pLSNIL12 retained clonogenic potential similar to wild type cells (data not shown).

For early passage brain tumor cultures (Ed141.MEL, Ed147.BT, Ed149.BT), transduction with pLSNB70, pLSNGM1, pLSNBG9, or pLSNIL12 did not significantly affect *in vitro* growth rates compared to wild type cells (**Figure 5-6**). However, D54MG (an established, immortalized human glioma cell line) showed greater variability in growth rates following retroviral transduction with therapeutic genes. In particular, D54MG transduced with pLSNIL12 grew significantly slower than wild type D54MG (1.75×10^5 +/- 1.56×10^4 cells *vs.* 4.09×10^5 +/- 1.27×10^4 cells by day 9; $p < 0.005$).

Conclusions

The dismal prognosis associated with many brain tumors (3, 6) has prompted intensive investigation of novel therapeutic strategies (14, 30, 31). Immunogene therapy (vaccination with irradiated autologous tumor cells that have been genetically modified to increase their immunogenicity) is one such strategy that has produced dramatic results in pre-clinical models (5, 22, 24, 25, 29). However, prior to embarking on immunogene therapy clinical trials, we wished to further characterize genetically altered human brain tumor cell cultures that might form the basis of these vaccines.

First, we demonstrated that our retroviral vectors could efficiently transfer immunostimulatory genes to cultured human brain tumor cells. This is not surprising given our previous report of B7-2 and GM-CSF transfer to D54MG (22). Indeed, we have successfully performed immunostimulatory gene transfer to 12 early passage melanoma cultures and 32 early passage glioma cultures (data not shown). More importantly, we demonstrated that irradiation with 200 Gy does not affect B7-2, GM-CSF, or IL-12 expression in appropriately transduced cultures (**Figures 5-2 and 5-3, Table 5-1**). This is a critical finding because if irradiation altered therapeutic gene expression, clinical vaccine doses would need to be altered accordingly.

In order to prevent any possibility of secondary tumor growth at vaccination sites, tumor cell vaccines are irradiated to prevent further cell division prior to administration subcutaneously. In previous vaccine trials with irradiated glioma cells, a dose of 200 Gy has been commonly used for this purpose (17). As it was theoretically possible that our genetically altered tumor cells could have altered sensitivity to ionizing radiation, we wished to examine their viability and clonogenicity after 200 Gy. As expected, viability is briefly maintained then rapidly drops off after irradiation (**Figure 5-4**). Clonogenicity is completely abolished suggesting that no further cell division takes place (**Figure 5-5**). This data is reassuring as it suggests that the possibility of secondary tumor growth at vaccine sites is minimal. The rapid drop off in viability underscores the transient nature of immunogene therapy vaccines and the need for multiple vaccinations.

Most early passage human brain tumor cultures grow considerably slower than established immortalized cell lines (20). In our experience, creating autologous tumor cell vaccines via retroviral gene transfer requires several months. Therefore, establishing the effect of retroviral transduction with B7-2, GM-CSF, and IL-12 genes on *in vitro* growth rates was critical. For example, if one gene product consistently reduced growth rates, preparation of autologous tumor cell vaccines transduced with this gene might not be feasible. In the present study, none of the early passage cultures (Ed141.MEL, Ed147.BT, Ed149.BT) showed any evidence of altered growth rates after transduction with immunostimulatory genes. However, D54MG (an immortalized human glioma cell line) showed more variation and appeared to be significantly inhibited by IL-

12 gene transfer (**Figure 5-6**). It is not immediately clear why this is the case. Possibly, pLSNIL12-transduction's inhibitory effects are only seen in very rapidly dividing cell lines such as D54MG and not in more slowly dividing early passage cultures. Regardless, the fact that early passage cultures are unaffected is reassuring as it suggests that expanding these cells in culture to sufficient numbers for clinical purposes is feasible.

In addition to the characteristics examined here, there may be many other aspects of these cultures of immunologic importance. For example, it is possible that irradiation affects tumor cells' ability to present antigen to T cells. This could significantly impair the ability of these vaccines to stimulate immune responses. Ultimately, the most important characteristic of these vaccine preparations is their ability (or lack thereof) to stimulate anti-tumor immune responses. While we have not examined this in the present study, we have previously demonstrated that vaccination with irradiated human glioma cells expressing B7-2 and GM-CSF generates a systemic response that inhibits growth of pre-established human glioma xenografts in a human lymphocyte / severe combined immunodeficient mouse model (22).

In summary, this study demonstrates that B7-2, GM-CSF, and IL-12 genes can be efficiently transferred to cultured human brain tumor cells using our retroviral vectors. Irradiation (200 Gy) does not significantly alter expression of these genes in transduced cells. Cells remain viable for several days following irradiation, but this drops off quickly and most cells are dead by day 10. Irradiated cells are no longer clonogenic, suggesting that they are incapable of further cell division. Early passage brain tumor culture growth rates *in vitro* are not altered after therapeutic gene transfer. These results suggest that creating vaccines with irradiated autologous brain tumor cells that have been genetically altered to increase their immunogenicity is feasible and provide some basic safety information about these cells.

Table 5-1. Expression of therapeutic genes

Expression of therapeutic genes in wild type and transduced cells with or without radiation (200 Gy). B7-2 expression (based on flow cytometry) is either present or absent. GM-CSF and IL-12 expression is measured in ng / 10^6 cells / 24 hours. N.D. = Not Done.

Table 5-1.

Cell Line	Retrovirus	Irradiation	B7-2	GM-CSF	IL-12
D54MG	None	No	No	0.00	0.38
D54MG	pLSNB70	No	Yes	N.D.	N.D.
D54MG	pLSNGM1	No	N.D	31.17	N.D.
D54MG	pLSNIL12	No	N.D	N.D.	1.30
D54MG	pLSNBG9	No	Yes	7.79	N.D.
D54MG	None	Yes	No	0.00	0.53
D54MG	pLSNB70	Yes	Yes	N.D.	N.D.
D54MG	pLSNGM1	Yes	N.D	76.92	N.D.
D54MG	pLSNIL12	Yes	N.D	N.D.	1.60
D54MG	pLSNBG9	Yes	Yes	N.D.	N.D.
Ed141.MEL	None	No	No	0.00	0.38
Ed141.MEL	pLSNB70	No	Yes	N.D.	N.D.
Ed141.MEL	pLSNGM1	No	N.D	22.34	N.D.
Ed141.MEL	pLSNIL12	No	N.D	N.D.	1.30
Ed141.MEL	pLSNBG9	No	Yes	1.24	N.D.
Ed141.MEL	None	Yes	No	0.20	0.53
Ed141.MEL	pLSNB70	Yes	Yes	N.D.	N.D.
Ed141.MEL	pLSNGM1	Yes	N.D	28.12	N.D.
Ed141.MEL	pLSNIL12	Yes	N.D	N.D.	1.60
Ed141.MEL	pLSNBG9	Yes	Yes	N.D.	N.D.
Ed147.BT	None	No	No	0.22	N.D.
Ed147.BT	pLSNB70	No	Yes	N.D.	N.D.
Ed147.BT	pLSNGM1	No	N.D	9.80	N.D.
Ed147.BT	pLSNIL12	No	N.D	N.D.	N.D.
Ed147.BT	pLSNBG9	No	Yes	1.97	N.D.
Ed147.BT	None	Yes	No	0.23	
Ed147.BT	pLSNB70	Yes	Yes	N.D.	N.D.
Ed147.BT	pLSNGM1	Yes	N.D	6.28	N.D.
Ed147.BT	pLSNIL12	Yes	N.D	N.D.	
Ed147.BT	pLSNBG9	Yes	Yes	3.77	N.D.
Ed149.BT	None	No	No	0.35	0.00
Ed149.BT	pLSNB70	No	Yes	N.D.	N.D.
Ed149.BT	pLSNGM1	No	N.D	46.70	N.D.
Ed149.BT	pLSNIL12	No	N.D	N.D.	2.10
Ed149.BT	pLSNBG9	No	Yes	2.90	N.D.
Ed149.BT	None	Yes	No	0.13	0.00
Ed149.BT	pLSNB70	Yes	Yes	N.D.	N.D.
Ed149.BT	pLSNGM1	Yes	N.D	61.00	N.D.
Ed149.BT	pLSNIL12	Yes	N.D	N.D.	1.17
Ed149.BT	pLSNBG9	Yes	Yes	3.72	N.D.

Figure 5-1.

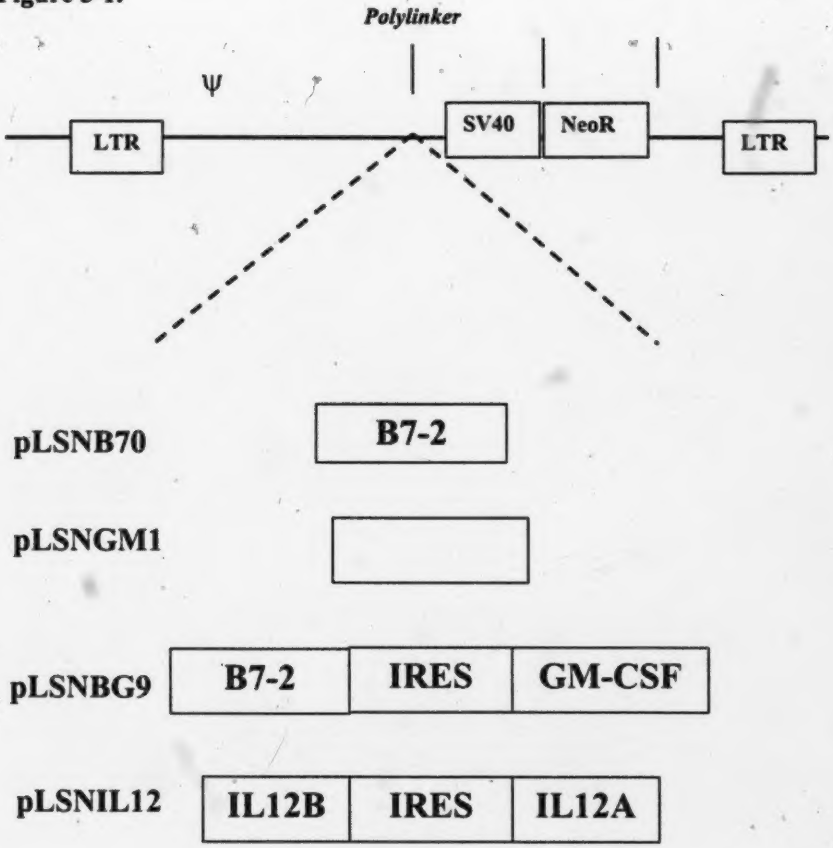
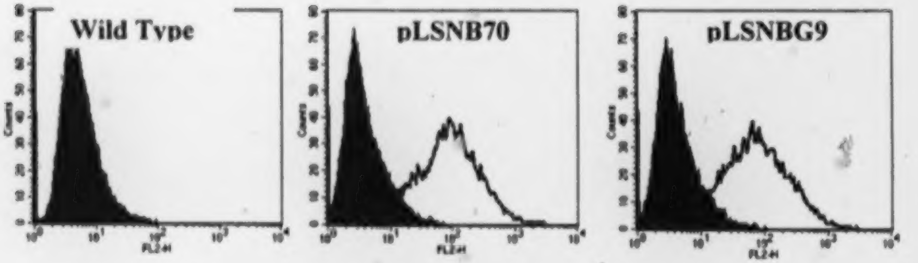

Polylinker
Ψ
LTR
SV40
NeoR
LTR
pLSNB70
B7-2
pLSNGM1
pLSNBG9
B7-2
IRES
GM-CSF
pLSNIL12
IL12B
IRES
IL12A

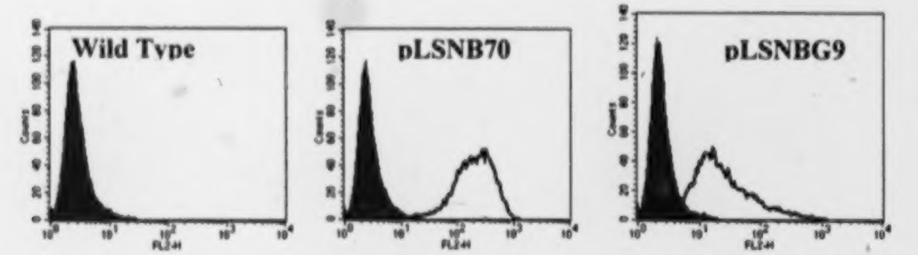
Figure 5-1. Retroviral Plasmid Maps

Schematic plasmid maps showing retroviral vectors encoding B7-2, GM-CSF, B7-2 and GM-CSF, and IL-12. All therapeutic genes are under the control of an LTR promoter region (in pLSNBG9 and pLSNIL12, two therapeutic genes under the control of this single promoter are linked by an internal ribosome entry site - IRES). All vectors contain a neomycin resistance gene under the control of a separate SV40 promoter region. All lack retroviral *gag, pol,* and *env* genes, rendering them replication-incompetent.

Figure 5-2.



A. Unirradiated



B. Irradiated

Figure 5-2. B7-2 Expression + / - Irradiation

Representative flow cytometry histograms showing B7-2 expression in D54MG cells (wild type, pLSNB70-transduced, and pLSNBG9-transduced) before (A) and after (B) irradiation (200 Gy). Shaded histograms represent background fluorescence when stained with an isotype-matched control antibody; dark single line histograms represent fluorescence when stained with a monoclonal antibody specific for human B7-2 (CD86).

Figure 5-3.

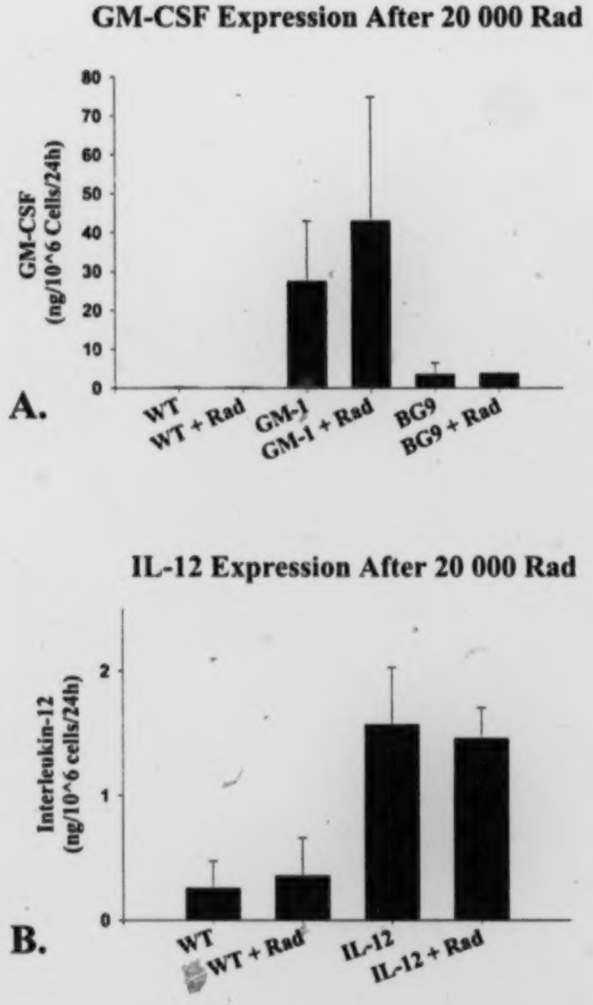


Figure 5-3. GM-CSF and IL-12 Expression + / - Irradiation

GM-CSF (A) and IL-12 (B) expression levels in wild type (WT), pLSNGM-1-transduced (GM-1), pLSNBG9-transduced (BG9), and pLSNIL12-transduced (IL-12) brain tumor cultures with and without irradiation (200 Gy). Values represent mean +/- s.e.m. for pooled data from Ed141.MEL, Ed147.BT, Ed149.BT, and D54MG.

Figure 5-4.

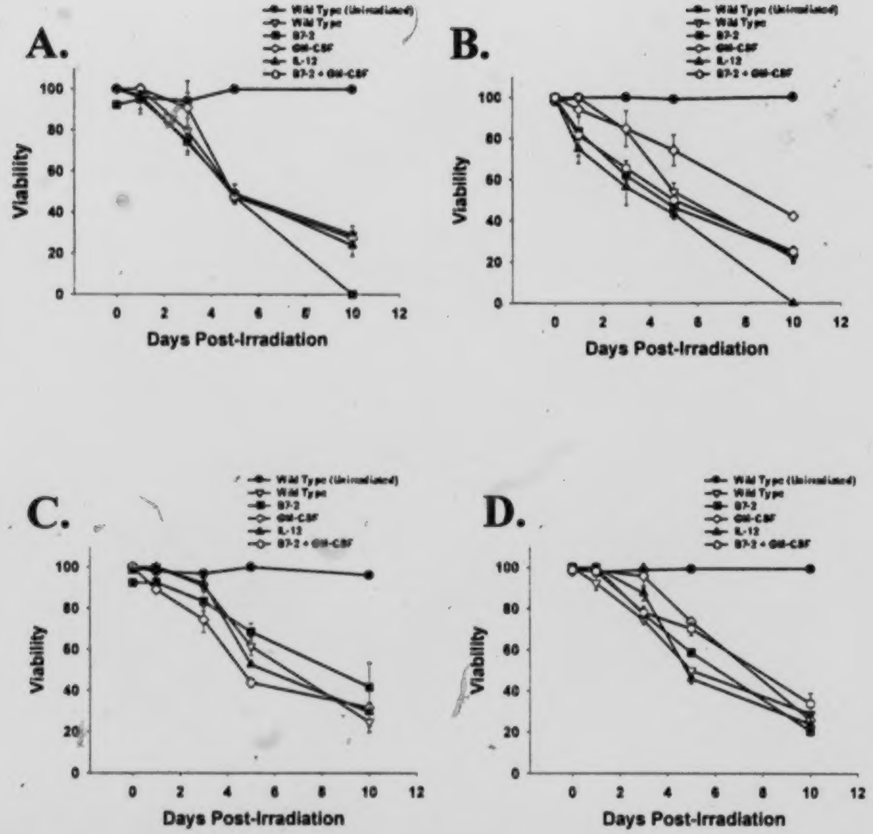


Figure 5-4. Viability Curves Following Irradiation

Viability curves following irradiation (200 Gy) for (A) Ed141.MEL, (B) Ed147.BT, (C) Ed149.BT, and (D) D54MG. Cells were irradiated on day 0. Values represent mean +/- s.d. for assays performed in triplicate. Unirradiated wild type cells were used as controls in each case.

Figure 5-5.

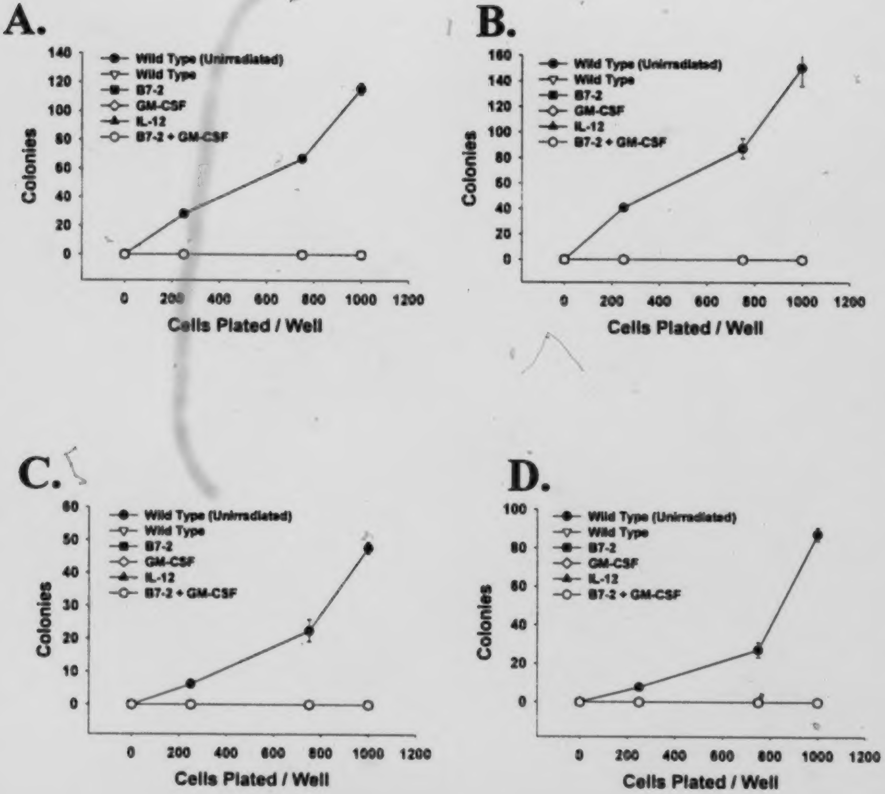


Figure 5-5. Clonogenicity Curves Following Irradiation

Clonogenicity curves following irradiation (200 Gy) for (A) Ed141.MEL, (B) Ed147.BT, (C) Ed149.BT, and (D) D54MG. Cells were plated out in 6 well plates following irradiation at 0, 750, and 1000 cells/well. Values represent mean +/- s.d. for assays performed in triplicate. Unirradiated wild type cells were used as controls in each case.

Figure 5-6.

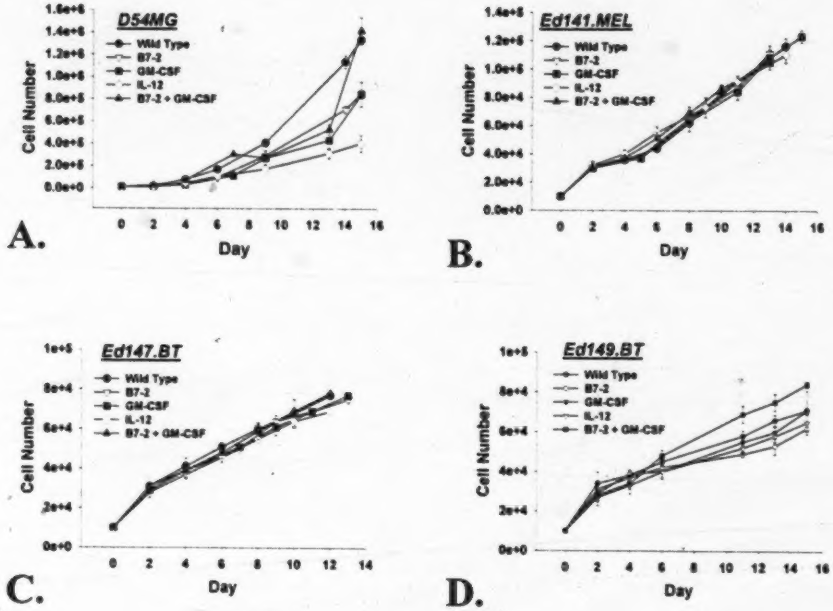


Figure 5-6. Growth Curves After Therapeutic Gene Transfer

In vitro **growth curves after pLSNB70, pLSNGM1, pLSNBG9, and pLSNIL12-transduction for (A) Ed141.MEL, (B) Ed147.BT, (C) Ed149.BT, and (D) D54MG. Cells were initially plated out in 24 well plates at 5 x 10^3 cells/well. Values represent mean +/- s.d. for assays performed in duplicate.**

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Chapter 6

GM-CSF and B7-2 Combination Immunogene Therapy in an Allogeneic hu-PBL-SCID Mouse / Human Glioblastoma Multiforme Model

A version of this chapter has been published previously. Parney et al. 1997. Human Gene Therapy. 8: 1073 - 1085.

Introduction

Despite improvements in diagnosis and treatment of glioblastoma multiforme, mean survival from time of diagnosis remains less than one year (14, 52). However, recent advances concerning the molecular biology of this tumor (45, 50, 79) have led to study of novel molecular treatment methods, including gene therapy (41, 86). It is hoped that these new modalities will eventually lead to improved survival for glioblastoma patients.

Recent glioblastoma gene therapy studies have concentrated on *in vivo* gene transfer using the HSV-tk / ganciclovir paradigm (21, 22, 58, 61, 62, 72, 73). While this system shows some promise, the biodistribution of gene therapy vectors carrying the HSV-tk gene *in situ* remains problematic. Quoted transduction rates vary widely from 10 to 70% (3, 21, 62, 74). Despite the observed "bystander" killing effect that extends to neighboring untransduced cells (29, 80, 81), subtotal gene transfer still limits the efficacy of this approach.

To overcome such problems, alternative gene therapy strategies have been developed. Vaccination with irradiated tumor cells genetically modified to increase their immunogenicity has inhibited local and systemic tumor growth in several animal tumor models (24, 39, 67) and has now proceeded to clinical trial in the treatment of renal cell carcinomas (8). Unlike HSV-tk/ganciclovir therapy, this approach employs *in vitro* immunogene transfer followed by immunization with irradiated, gene-modified tumor cells. This avoids subtotal gene expression as transduced cells can be selected *in vitro* prior to immunization via co-transduced antibiotic resistance genes.

Immunogene therapy for glioblastoma has been reported in a small number of studies involving animal models (26, 35, 85) and in one clinical case report (71). These reports suggest the potential susceptibility of glioblastoma tumors to immunogene therapy and are consistent with known glioblastoma immunobiology. Although glioblastomas *in situ* are normally infiltrated to varying degrees by lymphocytes (9, 42), evidence indicates these lymphocytes are unactivated. This may be in part due to secretion of immunosuppressive factors such as prostaglandin E_2, transforming growth factor β_2, and interleukin-10, all of which inhibit lymphocyte activation (27, 38, 42, 57, 68, 69). Immunogene therapy strategies are designed to overcome such local immunosuppression by promoting tumor antigen presentation and/or anti-tumor lymphocyte activation.

Many immunomodulatory genes are potentially useful and more than one may be necessary for overcoming tumor immunosuppression. These include genes encoding cytokines, major histocompatibility complex molecules, and T cell costimulatory molecules (24, 31). An ideal tumor vaccine should coexpress a combination of immunostimulatory genes from distinct immunomodulatory pathways (e.g. costimulators, cytokines, and chemoattractive adjuvants). In this study, we have focused on two such genes: the therapeutic cytokine granulocyte-macrophage colony stimulating factor (GM-CSF) and the T-cell costimulatory molecule B7-2.

GM-CSF stimulates growth and differentiation of granulocytes, monocytes/macrophages, microglia, and other antigen presenting cells. It has recently come into widespread clinical use as a treatment of neutropenia due to its hematopoietic effects (1, 25, 46). The importance of this cytokine in tumor immunogene therapy was recently demonstrated by Dranoff, *et al.*. (24) who showed that vaccination with irradiated GM-CSF-transduced tumor cells produced specific and marked growth inhibition of wild type tumor challenges in mouse models of adenocarcinoma and melanoma. Of 10 cytokine genes tested, GM-CSF resulted in the greatest tumor growth inhibition.

B7-2 is one of a family (B7-1, B7-2, and B7-3) of lymphocyte cell surface molecules that have recently been identified as costimulatory molecules necessary for T-cell activation in conjunction with antigen presentation in the context of a major histocompatibility complex molecule (11, 13, 28, 47, 64). The absence of costimulatory molecules on tumor cells may contribute to their failure to be detected and eliminated by the immune system (32). The specific roles of the various costimulatory molecules are yet to be clearly defined. It has been suggested that B7-1 expression promotes differentiation of intermediate T_H cell precursors into T_H1 effector cells (cellular immune responses) while B7-2 expression leads to T_H2 differentiation (humoral immune responses) (40, 76). However, both B7-1 and B7-2 have been shown to promote cell mediated immune responses in animal models (37, 43, 59). More recently, the effectiveness of B7-1 expression in promoting tumor rejection has been questioned (82). Furthermore, T cells must receive costimulatory signals from antigen-presenting cells (APC's) within the first 12 hours of T cell receptor stimulation for maximal interleukin-2 production (54). Therefore, the rapid induction of B7-2 (not B7-1) on APC's after antigen stimulation suggests that B7-2 is the preferable costimulatory molecule to promote antitumor cell-mediated immune responses (32).

Most preclinical immunogene therapy studies have used rodent genes and rodent tumor models. However, the applicability of such models to human systems is unclear. This is particularly true for glioblastoma models utilizing rodent tumors (rat 9L-glioma and C6-glioma) which have sarcomatous features that are significantly different from human glioblastoma multiforme(4, 7, 23). Therefore, we have develped a humanized mouse model utilizing a human glioblastoma cell line, human immunogenes, and human lymphocytes.

Using retroviral vectors previously modified in our laboratory (65), we examined the efficacy of human GM-CSF and/or B7-2 gene transfer *in vitro* into a human glioblastoma cell line. Thereafter, the effect of GM-CSF and/or B7-2 expression on glioblastoma growth *in vivo* was examined in a human tumor/human peripheral blood lymphocyte /severe combined immunodeficiency mouse (hu-PBL-SCID) model. Human glioblastoma cells (with or without therapeutic gene transfer) and human peripheral blood lymphocytes were grafted into SCID/bg or SCID/nod mice and the effect on tumor growth locally and at distant sites was observed. SCID/bg and SCID/nod mice can accept human tumor and lymphocyte grafts without rejection as they lack mature T, B, and NK lymphocytes (10, 15, 16, 20, 34, 48, 49, 51, 55, 60). In this initial study, we used an allogeneic system with an established human glioblastoma cell line and lymphocytes from unrelated donors. Our primary objectives included establishing the feasibility of immunogene therapy for glioblastoma and developing a preclinical *in vivo* model of human glioblastoma immunology.

Methods

Growth of D54MG in Tissue Culture

The human glioblastoma cell line D54MG was cultured in Dulbucco's Modified Eagle's Media (DMEM) with 10% fetal bovine serum (Gibco), 0.2 units/ml penicillin-streptomycin (Sigma) solution, and 0.2 mM glutamate at 37°C with 5% CO_2.

Preparation of Retroviral Vectors

GM-CSF and B7-2 cDNAs were amplified from normal human lymphocyte RNA by rtPCR. The cDNAs were first ligated into the bacterial plasmid pBluescript KS (-) (Stratagene) and subsequently into the retroviral gene therapy vector pLSN which was derived from modified murine Moloney leukemia virus (MLV) (65). In addition, cDNA for the reporter gene Green Flourescent Protein (GFP, Clontech) was also inserted into the retroviral vector pLSN. All the vectors contained a neomycin resistance gene driven by an internal SV40 promoter. Retroviral plasmid DNA was transfected into the packaging cell line PA317 by lipofection as previously described (65). Replication incompetent virus was harvested from supernatant of the transfected PA317 cultures and immediately frozen at -80°C for later use.

Retroviral Transduction

Virus stock was thawed from -80°C at 37°C. Polybrene was added to the thawed virus solution at a final concentration of 4 μg/ml. Culture media was added to the virus solution to bring the final volume to 1.5 ml. Logarithmic growth phase D54MG cells in T25 flasks were incubated in the virus supernatant at 37°C, 5% CO_2 for 3 hours. The same volume of media and polybrene without virus was used as control. After 3 hours, a further 3 ml of culture media was added to each flask and they were incubated overnight. Media was changed the next morning.

Selection of Transduced Cells

Twenty-four hours after retroviral transduction, 500 μg/ml of the neomycin analogue G418 was added to culture media. Media was changed every two to three days until complete selection had taken place (i.e. all cells in the control flasks were dead). After selection, the cells were cultured in growth media containing reduced concentrations of G418 (250 μg/ml) and allowed to grow to confluence.

Analysis of B7-2 Expression by Flow Cytometry

Transduced D54MG cells at approximately 70% confluence were harvested by scraping after room temperature incubation for 15 minutes in 0.02% EDTA in Phosphate-Buffered Saline (PBS). In aliquots of 10^6 cells per 200 μl of immunofluoresence (IF) buffer (2% fetal calf serum, 0.02% sodium azide in PBS), samples were incubated on ice for 1 hour with 1 μg of RPE-conjugated monoclonal anti-human B7-2 antibody (Ancell) or 1 μg of RPE-conjugated isotype-control murine IgG_1 antibody (Pharmingen). Cells were washed four times in IF buffer, and fixed in 1% Formalin in PBS. Samples were then read on a B-D Flow Cytometer as per standard techniques.

Analysis of GM-CSF Expression by ELISA

Transduced D54MG cells at approximately 70% confluence in T75 flasks were incubated in fresh media for 24 hours. After this period of incubation, media was harvested and centrifuged briefly to remove cells and debris. Cell number per flask was counted. GM-CSF levels in the harvested media was tested using a commercially available kit (Quantikine, R & D Systems, California) as per the manufacturer's instructions. These levels were converted to $\mu g/10^6$cells/24 hours based on the cell number in the flasks from which the media originated.

Animals and human PBL reconstitution

For most experiments, four to five week old female C.B-17-SCID-beige mice were purchased from Taconic (Germantown, New York). For the first vaccination/challenge experiment, four to five week old female SCID/nod mice were obtained from Dr. L. Pilarski (Cross Cancer Institute, University of Alberta, Edmonton, Alberta). The mice were maintained in filtered cages in a virus free environment and received cotrimoxazole in their drinking water twice per week.

Hu-PBL-SCID mouse reconstitution was carried out as previously described (88). Briefly, each mouse was intraperitoneally (i.p) injected with 2-3 x 10^7 PBLs resuspended in 0.5 ml of Hanks' balanced salt solution. A near 100% success rate in SCID/bg mice had been obtained when fresh PBLs were used. Five days to three weeks after reconstitution, mice were bled from the tail and the human Ig level was assessed by enzyme-linked immunosorbent assay (ELISA) using a monoclonal rabbit anti-human IgG/IgM antibody (Jackson Labs) and control human IgG (Sigma). Our preliminary results indicated that SCID/nod mice were not as reliably reconstituted with human peripheral blood lymphocytes as SCID/bg mice. Unlike SCID/bg mice, reconstitution of SCID/nod mice with human PBL's appeared to vary both from PBL donor to PBL donor and from mouse to mouse. Furthermore, many hu-PBL-SCID/nod mice developed a disease characterized by cachexia, alopecia, and facial edema 3 to 6 weeks after reconstitution. This was not seen in unreconstituted SCID/nod mice or in SCID/bg mice with or without reconstitution. The etiology was unclear, though the possibilities of Graft *vs.* Host Disease and diabetes mellitus were considered. Because of these difficulties, most experiments were performed with SCID/bg mice.

Subcutaneous Tumor Growth Experiments

Five to seven week old SCID/bg mice were injected subcutaneously on the right flank with 2 x 10^6 retrovirally-transduced and selected D54MG cells. Control mice were injected subcutaneously on the right flank with wild type D54MG cells. Six days post injection, mice were reconstituted via intraperitoneal injection with 2 x 10^7 human peripheral blood lymphocytes isolated on a Ficoll-Paque gradient (Sigma) from the buffy coat layer of whole blood from healthy donors as described above. Reconstitution was monitored by tail blood sera ELISA's for human immunoglobulin. In all but one experiment, half of the mice from each group (transduced and untransduced) were left unreconstituted as controls. Tumor size was measured in three directions by calipers every 3 to 5 days. Comparison was made between transduced and untransduced tumors and between reconstituted and unreconstituted mice.

Vaccination / Challenge Experiments

In the first vaccination/challenge experiment, five to six week old female SCID/nod mice were reconstituted with 2 x 10^7 PBL from healthy donors via intraperitoneal injection. Reconstitution was monitored by tail blood sera ELISA's for human immunoglobulin. Five days after reconstitution, mice were vaccinated via intraperitoneal injection of 1 x 10^5 irradiated (20,000 rad) D54MG cells (either wild type, GFP-transduced, or B7-2 and GM-CSF-transduced). Five days post-vaccination, all mice received subcutaneous injections of 1 x 10^6 unirradiated wild type D54MG cells on the right flank. Tumor growth was measured serially as above.

The second vaccination experiment was performed similarly, but in this instance, SCID/bg mice (Taconic) were used. In addition, the order of injections was slightly different. These mice first received subcutaneous injections of 1 x 10^6 wild type D54MG cells on their right flanks. Ten days later, they were reconstituted with 2 x 10^7 human PBL. Ten days following this, they were vaccinated with 1 x 10^5 irradiated tumor cells (either wild type, GFP-transduced, or B7-2/GM-CSF-transduced).

Statistical Analysis

Comparison between tumor sizes in different groups was performed using standard one-way analysis of variance (ANOVA).

Results

Construction of Mono- and Bi-cistronic Retroviral Vectors

Retroviral gene therapy vectors were constructed that contained the genes for GM-CSF, B7-2, GM-CSF and B7-2, or GFP gene as shown in **Figure 6-1**. All genes were cloned into the MLV-based pLSN plasmid (65). Therapeutic genes were inserted into a region under the control of the retroviral LTR (long terminal repeat) whereas a neomycin-resistance gene was under the control of an internal SV 40 promotor. The vectors lacked the gag, pol, or env genes necessary for retroviral packaging in order to render them replication incompetent. These structural proteins were provided in *trans* by the retroviral packaging cell line PA317. For the bi-cistronic vector containing GM-CSF and B7-2 genes, the two genes were interposed with an internal ribosome entry site (IRES) derived from the Encephalomyocarditis Virus genome (Novagen).

Expression of B7-2 and GM-CSF in the Human Glioblastoma Cell Line D54MG

Flow cytometry studies showed expression of B7-2 surface antigen on B7-2- and B7-2/GM-CSF-transduced D54MG cells but not on wild type or GM-CSF-transduced cells (1-2 orders of magnitude flourescence shift compared to isotype controls). Flow cytometry of GFP-transduced D54MG cells without staining revealed mildly increased autoflourescence compared to wild type D54MG. Flow cytometry histograms are shown in **Figure 6-2**. GM-CSF production was significant for GM-CSF-transduced (30 ng/10^6 cells/day) and B7-2/GM-CSF-transduced (5 ng/10^6 cells/day) cells but not for wild type or B7-2-transduced cells. Gene expression in transduced D54MG is summarized in **Table 6-1.**

Tumor Growth Efficiency and Human PBL Reconstitution in SCID/beige and SCID/nod Mice

Previous studies in our laboratory indicated that SCID/bg mice receiving human PBL via intraperitoneal injection supported the growth of a human melanoma cell line with nearly 100% success and that these animals demonstrated significant levels of human lymphocytes by flow cytometric analysis in spleen and peripheral blood 38 days post reconstitution (unpublished, Zhang *et al.*). In this study, both SCID/nod and SCID/bg mice supported the growth of wild type D54MG (human glioblastoma) subcutaneously with 100% efficiency (35/35) regardless of human lymphocyte reconstitution. D54MG tumors transduced with B7-2, GM-CSF, or both also grew with 100% efficiency in unreconstituted SCID/bg mice (4/4, 8/8, and 4/4 respectively). Growth of D54MG tumors transduced with B7-2 and GM-CSF was inhibited in human lymphocyte-reconstituted mice as detailed below.

Reconstitution with human lymphocytes was examined by ELISA of peripheral blood sera for human immunoglobulin. All SCID/bg mice receiving human lymphocytes (46/46) had significant levels (>100 μg/ml) of human immunoglobulin in serum within 14 days of reconstitution. However, 3 of 12 SCID/nod mice that received human lymphocytes failed to demonstrate human immunoglobulin on serial testing and were excluded from the study. Subsequent studies with SCID/nod mice revealed that success of human lymphocyte reconstitution also varied significantly from PBL donor to PBL donor (data not shown). For these reasons, SCID/bg mice (not SCID/nod) were primarily used in this study.

Growth Suppression of B7-2 and GM-CSF-Transduced D54MG but not Wild Type D54MG in Hu-PBL-SCID/bg Mice

In two separate experiments, growth of D54MG transduced with B7-2 in human lymphocyte reconstituted SCID/bg mice was markedly inhibited compared to untransduced and/or unreconstituted controls (**Figure 6-3**). A standard test for statistical significance in subcutaneous tumor growth models is comparison of tumor volumes by ANOVA at a point approximately two-thirds along the growth curve (33). By this criteria, the mean tumor volume for B7-2-transduced tumors in human lymphocyte-reconstituted mice was significantly less than wild type tumors by 22 days in the first experiment and by 35 days in the second experiment ($P<0.05$ in both). Interestingly, growth of D54MG-B7-2 tumors in unreconstituted mice was also mildly inhibited compared to wild type tumors (**Figure 6-3B**). However, growth inhibition was much more marked in reconstituted mice. This suggests that, although a small portion of the growth inhibition seen for D54MG-B7-2 may be human lymphocyte-independent, the predominant effect is dependent on human lymphocytes.

Growth of GM-CSF-transduced D54MG in human lymphocyte reconstituted mice was moderately inhibited compared to untransduced and/or unreconstituted controls in two separate experiments (**Figure 6-4**). Neither of these experiments achieved statistical significance by ANOVA on their own. However, when the results from the two experiments were pooled, GM-CSF-transduced tumors in human PBMC-reconstituted mice were significantly smaller than wild type by 55 days ($P<0.001$). There was no inhibition of D54MG-GM-CSF tumors in unreconstituted mice.

Efficacy of Immunization of hu-PBL-SCID/nod and hu-PBL-SCID/bg Mice with Therapeutic Gene-Modified D54MG

Growth of wild type D54MG tumors in human PBL-reconstituted SCID/nod mice which had been vaccinated with irradiated B7-2/GM-CSF-transduced D54MG was markedly inhibited compared to mice vaccinated with wild type or GFP-transduced D54MG (**Figure 6-5A**). However, small sample sizes due to the exclusion of several unsuccesfully reconstituted mice prevented this effect from reaching statistical significance. This problem was subsequently overcome by performing all remaining studies with reconstituted SCID/bg mice.

In a second vaccination experiment, wild type growth inhibition was seen in human PBL-reconstituted SCID/bg mice vaccinated with irradiated D54MG-B7-2/GM-CSF cells but not in mice vaccinated with either wild type D54MG or D54MG-GFP cells (**Figure 6-5B**). Unlike the first vaccination, wild type D54MG cells were injected subcutaneously into the flank 20 days prior to vaccination. At the time of vaccination, tumors were palpable (1-2 mm^3) on all mice. After vaccination, tumor growth continued exponentially in mice vaccinated with wild type or GFP-transduced D54MG. However, in mice vaccinated with GM-CSF/B7-2 transduced D54MG, tumor size increased slightly after vaccination (peaking at a mean of 8 mm^3) and then regressed. Differences between the experimental and control groups achieved statistical significance by ANOVA ($p<0.001$) by day 42.

Conclusions

Therapeutic gene expression in the D54MG human glioblastoma cell line is high after *in vitro* transduction with our retroviral vectors. Levels of GM-CSF production are comparable to those reported in other retrovirally transduced tumor cell lines (24, 39). Quantification of B7-2 expression is difficult using flow cytometry as expression is essentially a binary system (either present or absent on the cell surface). However, B7-2 molecules are clearly present on the surface of D54MG cells transduced with retroviral vectors containing the B7-2 gene and absent on wild type or GM-CSF-transduced cells. We conclude that the retroviral vectors used in this study are simple, reliable tools for transferring GM-CSF and/or B7-2 genes into human glioblastoma cells *in vitro*.

The results in the allogeneic animal model are promising. Inhibition of GM-CSF and B7-2-transduced tumors seen in human lymphocyte-reconstituted SCID/bg mice (**Figures 6-3 and 6-4**) suggests that expression of these genes by glioblastoma cells overcomes local immunosuppression and results in a significant antitumor immune response. Furthermore, inhibition of wild type challenge growth in mice vaccinated with irradiated tumor cells transduced with B7-2 and GM-CSF (**Figure 6-5**) suggests that expression of these genes by glioblastoma cells induces a systemic immune response that inhibits tumor growth at distant sites.

Although cancer immunogene therapy is a relatively new field, several studies have already shown the efficacy of GM-CSF in stimulating anti-tumor immune responses when transduced into tumor cells. This cytokine has been shown in non-CNS animal tumor models (24) and in animal glioma models (84) to induce potent, specific anti-tumor immunity when transduced into tumor cells. Indeed, clinical trials of non-CNS cancer immunogene therapies using GM-CSF are now underway (8). Our study is the first *in vivo* demonstration of human GM-CSF immunogene therapy in a human glioblastoma model.

T cell costimulatory molecules have also been previously proposed for immunogene therapy. Studies have examined B7-1 either alone (17, 18, 30, 32, 78), in combination with Class II MHC molecules (5, 6), or in combination with cytokines (interleukin-2, interleukin-6 and/or interleukin-12) (31, 66). These studies all demonstrated induction of anti-tumor immune responses after transfer of the B7-1 gene to tumor cells, though (as noted) Class II MHC molecules or cytokines were sometimes necessary.

Only three previous reports have examined cancer immunogene therapy using B7-2 gene transfer. Hodge, *et al.* (37) demonstrated that transfer of either B7-1 or B7-2 genes to a colon adenocarcinoma cell line via vaccinia virus vectors induced systemic anti-tumor immunity in syngeneic mice. Interestingly, Yang, *et al.* (83) showed that B7-2 gene transfer induced systemic anti-tumor immunity in syngeneic mice when transferred to an immunogenic mastocytoma cell line but not when transferred (either alone or in combination with B7-1) to a non-immunogenic fibrosarcoma cell line. The most recent study (19) demonstrated that, while transfer of B7-1 or B7-2 genes to melanoma and colorectal carcinoma cell lines resulted in a local anti-tumor response in syngeneic immunocompetent mice, a protective systemic immune response was absent and (for the melanoma cell line) may have attenuated the immune response compared to mice exposed initially to wild type tumor. These three studies all used the mouse B7-2 gene in a murine tumor models. Our results indicate that transfer of the human B7-2 gene markedly inhibited local glioblastoma growth (**Figure 6-3**) and (in combination with GM-CSF) resulted in marked inhibition of wild type glioblastoma growth at distant sites (**Figure 6-5**) in a human tumor-humanized mouse model. The reasons for the differing results of these studies are not immediately clear but may be related to methodological differences or to specific effects of B7-2 expression in different tumors. It has been proposed that the efficacy of B7-1 immunogene therapy is dependent on the underlying immunogenicity of the tumor (18). A similar effect may be seen for B7-2 gene transfer, as borne out by the results of Yang, *et al.* (83), but further studies are necessary.

We chose to combine GM-CSF and B7-2 in our immunogene therapy strategy because these two modulators activate distinctly different immunostimulatory pathways. Glioblastoma multiforme tumors contain highly heterogeneous cell populations. Because of this, it is probable that anti-tumor immune responses after transfer of any single gene will vary in glioblastoma cell lines derived from different patients (or even in cell lines derived from different portions of a single tumor). Thus, a "cocktail" of genes that stimulate different immunoreactive paths is more likely to be successful in inducing anti-tumor immunity in a glioblastoma patient than any single gene. Furthermore, although we cannot comment on synergism on the basis of our results to date, combination cytokine and T cell costimulatory molecule immunogene therapy has been shown to be synergistic (for interleukin-2 and B7-1 in the treatment of murine fibrosarcoma) in at least one previous report (66).

Although these results appear to indicate that immunogene therapy with B7-2 and GM-CSF induces systemic anti-tumor immune reactions against glioblastoma, it must be stressed that this work is derived from an allogeneic system and that the tumor is implanted at a different site from its tissue of origin. To clarify these issues, we are currently developing an intracranial hu-PBL-SCID mouse model utilizing autologous tumor cells and lymphocytes. However, the present model is closer to an autologous system than it may initially appear. Unlike the classical immune-mediated rejection seen in allogeneic organ transplantation, this system is free of graft-origin "passenger lymphocytes." These lymphocytes are important in initiating allogeneic organ rejection responses by presenting antigen to host lymphocytes in the context of allogeneic Class II Major Histocompatibility Complex (MHC) (12, 44, 53). The D54MG cell line expresses only Class I MHC and not Class II MHC *in vitro* (unpublished, Parney *et al.*.). Although the effect of allogeneic Class I MHC expression by D54MG cannot be denied, the absence of graft-origin Class II MHC-positive cells in the tumor renders this model more like an autologous system. However, the question of autologous *vs.* allogeneic responses can only be definitively answered with a truly autologous system.

The use of the hu-PBL-SCID mouse model and a human glioblastoma tumor cell line has generated promising *in vivo* preclinical results of immunogene therapy using GM-CSF or B7-2 genes (alone or in combination). This is a simple and powerful method to analyze human lymphocyte responses to human tumors *in vivo*. Early cancer immunotherapy studies using hu-PBL-SCID mice were limited by the influence of residual murine NK lymphocyte activity (36, 56, 63, 87). This problem has been overcome by the use of the SCID/bg and the SCID/nod mouse strains that are deficient in NK cells (20, 48, 49, 55, 60). While there remains some debate as to exactly how precisely (and for how long) hu-PBL-SCID mice simulate the human immune system, this model is gaining acceptance in the preclinical evaluation of human immunology *in vivo* (2, 34, 51, 70, 75, 77). Hopefully, continued application of this model system to cancer immunotherapy will reduce the difficulty in extrapolating data from preclinical animal models to clinical studies.

In conclusion, our retroviral gene therapy vectors demonstrated efficient bi-cistronic gene transfer (B7-2 and GM-CSF) to human glioblastoma cells *in vitro*. In an *in vivo* allogeneic human tumor/lymphocyte system, glioblastoma cells that express these genes result in lymphocyte-mediated responses that inhibit tumor growth locally and at distant sites. Thus, immunogene therapy for glioblastoma multiforme using B7-2 and GM-CSF genes is both feasible and promising. Further development of this model may provide valuable *in vivo* preclinical data to assess immunogene therapy strategies.

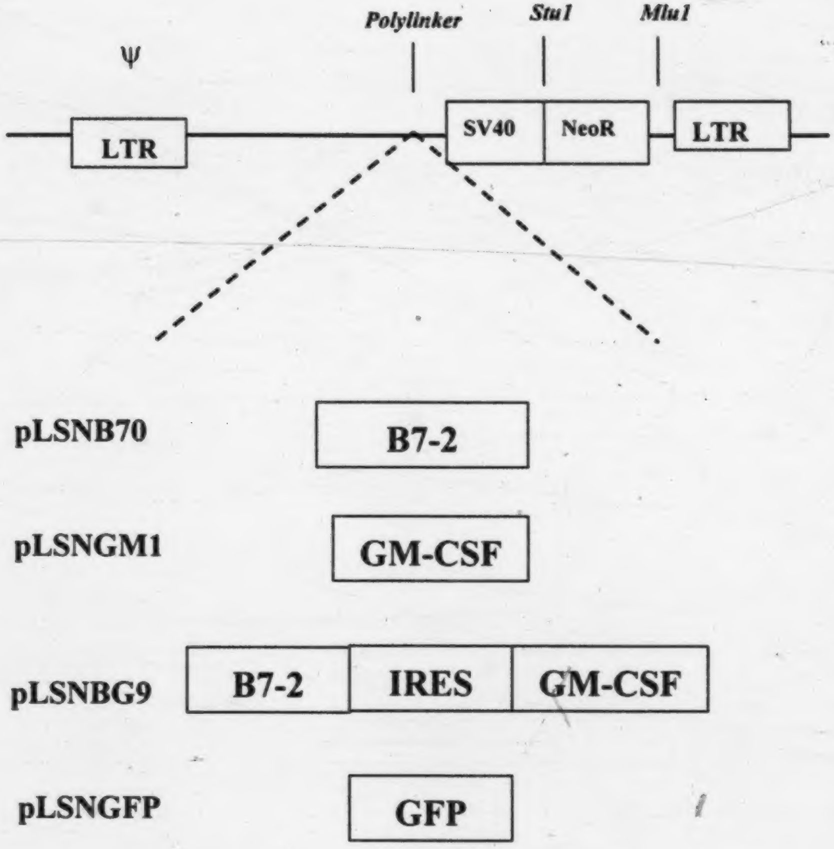
Table 6-1.

Cell Line	Vector (Genes Transferred)	GM-CSF Production ($ng/10^6$cells/day)	B7-2 Expression (orders of magnitude fluorescence shift)
D54MG	None	0.0	0
D54MG	pLSNB70 (B7-2)	0.0	2
D54MG	pLSNGM1 (GM-CSF)	30.0	0
D54MG	pLSNBG9 (B7-2 and GM-CSF)	5.0	1

Table 6-1. GM-CSF and B7-2 Expression

Therapeutic gene expression *in vitro* in wild type and transduced D54MG by ELISA (GM-CSF) or flow cytometry (B7-2).

Figure 6-1.



Polylinker
Stu1
Mlu1
ψ
LTR
SV40
NeoR
LTR
pLSNB70
B7-2
pLSNGM1
GM-CSF
pLSNBG9
B7-2
IRES
GM-CSF
pLSNGFP
GFP

Figure 6-1. Retroviral Plasmid Maps

Plasmid maps for replication-incompetent retroviral gene therapy vectors encoding: B7-2 (pLSNB70), GM-CSF (pLSNGM1), B7-2 and GM-CSF (pLSNBG9), and GFP (pLSNGFP).

Figure 6-2.

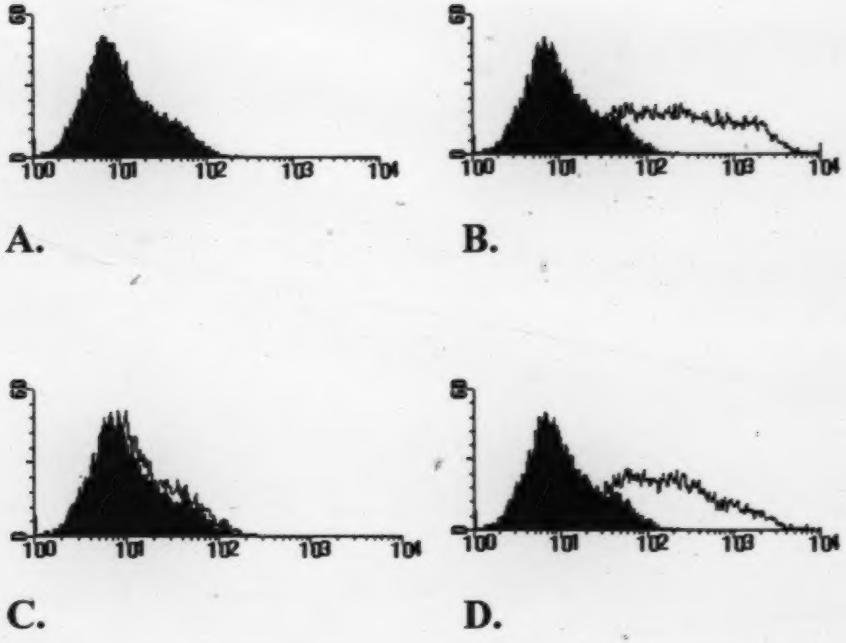


Figure 6-2. B7-2 Expression

Flow cytometry histograms for A) wild type D54MG, B) B7-2-transduced D54MG, C) GM-CSF-transduced D54MG, and D) B7-2 and GM-CSF-transduced D54MG. Shaded histograms represent isotype matched control antibodies while single dark line histograms represent monoclonal anti-human B7-2 antibodies.

Figure 6-3.

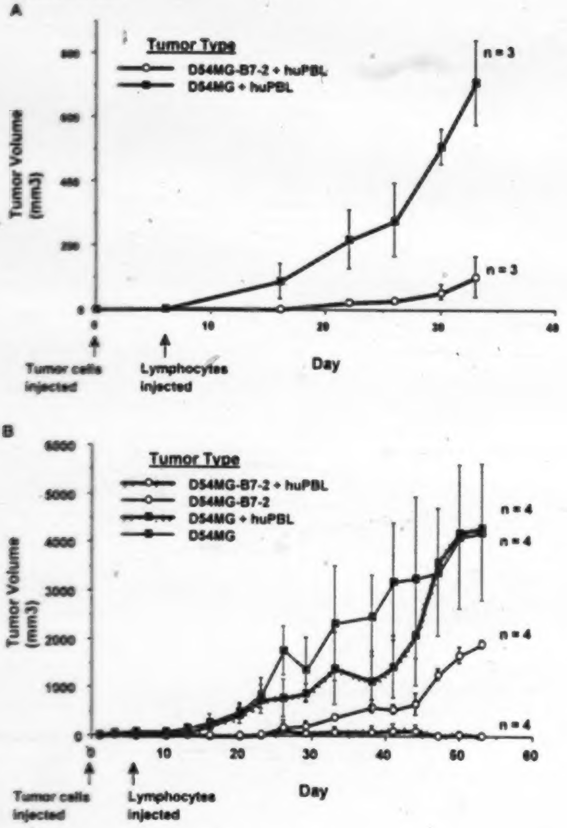


Figure 6-3. Growth of B7-2-Transduced Tumors

Inhibited growth of B7-2-transduced D54MG compared to wild type D54MG in hu-PBL-SCID/bg mice in two separate experiments (A and B). All mice received 2×10^6 tumor cells (either D54MG or D54MG-B7-2) subcutaneously on the right flank on Day 0. Mice were injected intraperitoneally with 2×10^7 human PBL six days after tumor cell injection. Reconstitution was confirmed by detecting serum human Ig levels > 100 µg/ml. In the first experiment (A), all mice were reconstituted. In the second experiment (B), half the mice from both groups (D54MG and D54MG-B7-2) were left unreconstituted (thin lines).

Figure 6-4.

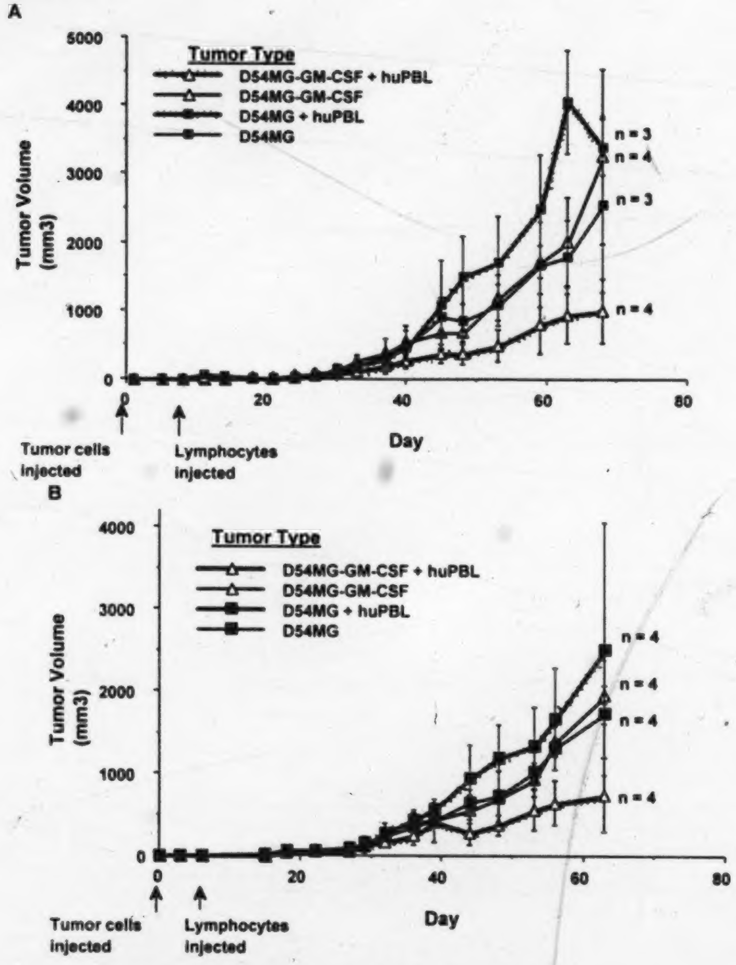


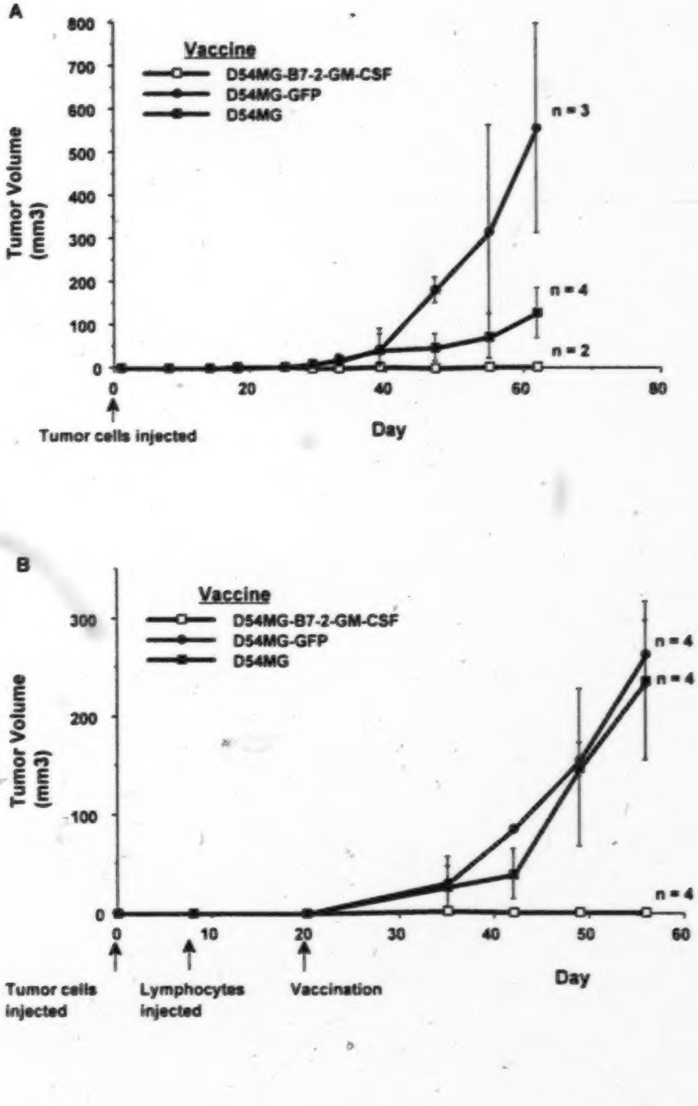
Figure 6-4. Growth of GM-CSF-Transduced Tumors

Inhibited growth of GM-CSF-transduced D54MG compared to wild type D54MG in hu-PBL-SCID/bg mice in two separate experiments (A and B). All mice received 2 x 10^6 tumor cells (either D54MG or D54MG-GM-CSF) subcutaneously on the right flank on Day 0. Mice were injected intraperitoneally with 2 x 10^7 human PBL six days after tumor cell injection. Reconstitution was confirmed by detecting serum human Ig levels > 100 µg/ml. In both experiments (A and B), half the mice from both groups (D54MG and D54MG-GM-CSF) were left unreconstituted (hatched lines).

Figure 6-5. Growth of Wild Type Tumors After B7-2 / GM-CSF-Transduced Vaccination

Inhibition of wild type D54MG challenges in human PBL-SCID/nod and SCID/bg mice vaccinated with irradiated D54MG-B7-2/GM-CSF cells. In the first experiment (A), SCID/nod mice were reconstituted with 2 x 10^7 human PBL via intraperitoneal injection 14 days prior to Day 0. They were vaccinated via intraperitoneal injection of 1 x 10^5 irradiated D54MG cells (either wild type, GFP-transduced, or B7-2/GM-CSF-transduced) 7 days prior to Day 0. On Day 0, they received 1 x 10^6 wild type D54MG cells subcutaneously on the right flank. Subsequent serial testing of tail blood for human immunoglobulin revealed that 3 mice (of 12 originally injected with human PBL) were not successfully reconstituted. These mice were excluded from the study and are not included in this data. The second experiment (B) was modified slightly from the first. SCID/bg mice were used instead of SCID/nod and the order of injections was changed. Wild type D54MG cells were injected on the flank on Day 0, mice were reconstituted with human PBL on Day 7, and mice were vaccinated on Day 20. All mice were successfully reconstituted with human lymphocytes by tail blood ELISA for human immunoglobulin (> 100 μg/ml).

Figure 6-5.


A
Vaccine
D54MG-B7-2-GM-CSF
D54MG-GFP
D54MG
Tumor Volume (mm3)
800
700
600
500
400
300
200
100
0
0
20
40
60
80
Day
Tumor cells injected
n = 3
n = 4
n = 2
B
Vaccine
D54MG-B7-2-GM-CSF
D54MG-GFP
D54MG
Tumor Volume (mm3)
300
200
100
0
0
10
20
30
40
50
60
Day
Tumor cells injected
Lymphocytes injected
Vaccination
n = 4
n = 4
n = 4

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Chapter 7

General Discussion and Conclusions

Introduction

Gliomas, the most common primary brain tumor, continue to be a devastating clinical problem despite advances in surgery, radiotherapy, and chemotherapy. Mean survival for patients with glioblastoma multiforme tumors remains less than one year, a figure that has not changed significantly in 3 decades (5, 10). Fortunately, advances in molecular biology have revolutionized the study of gliomas in the past 10 to 15 years and leading to many new experimental treatment modalities for gliomas (15, 32, 34). As a result of these advances, interest in immunotherapy (long investigated for glioma treatment but with regrettably poor results) is currently undergoing a renaissance. This thesis reflects this renewed interest.

Review of Data to Date

In our experiments, we chose to work exclusively with human gliomas rather than the more commonly studied rodent glioma models such as 9L gliosarcoma and C6 glioma. While these rodent tumors are well characterized and relatively easy to reproduce, they often exhibit features that are quite distinct from human gliomas (1, 2). However, human glioma cells are often quite difficult to culture (4, 18, 28). Therefore, we desired an improved method for establishing human glioma cell cultures to allow us to study human glioma cells more easily. As outlined in Chapter 2, we were successful in this endeavor. We found that centrifuging on a density gradient prior to plating glioma-derived single cell suspensions reduced the number of contaminating mesenchymal cells such as fibroblasts. This allowed us to increase our success rate for establishing human brain tumor cultures from 42% to 86% and set the stage for our further studies of human glioma immunology and immunogene therapy.

Prior to embarking on glioma immunogene therapy studies, we wished to clarify some aspects of human glioma immunobiology. Several recently published papers have reported dramatic results with immunogene therapy in animals harboring intracranial tumors. However, the efficacies of particular strategies appear to vary widely with the animal model chosen (8, 21, 22, 31). As it seemed likely that this reflected differences in basic immunobiology in different tumor models, we wished to examine aspects of human glioma immunobiology in order to predict more accurately which immunogene therapy strategies are likely to be effective against human gliomas. Furthermore, we felt that the molecular correlates of one of the basic assumptions of immunogene therapy (namely, that human gliomas are potentially sensitive to cell-mediated immune responses despite the fact that such responses to do not normally occur) had not been adequately investigated.

Therefore, we undertook a number of investigations into basic human glioma immunobiology as outlined in chapters 3 and 4. We found that human gliomas possess the basic machinery to be recognized (class I MHC) and killed

(Fas, in the absence of Fas-ligand) by cytotoxic T cells suggesting that they are potentially sensitive to the major mechanisms of cell-mediated immunity. However, they are not ideal antigen presenting cells as they lack class II MHC and T cell costimulatory molecules such as B7-2. Furthermore, they secrete immunoinhibitory factors such as TGF-β2 and PGE2 and T_H2 cytokines such as IL-6 that may inhibit activation of anti-tumor cell mediated immunity. Significant expression of immunostimulatory genes such as GM-CSF and IL-12 is lacking. These data help establish molecular correlates for the assumptions underlying glioma immunogene therapy. Based on this data, one can predict that transferring immunostimulatory genes such as class II MHC, B7-2, GM-CSF, or IL-12 to human glioma cells or inhibiting immunoinhibitory genes such as TGF-β2, PGE2, or IL-6 should stimulate anti-glioma cell-mediated immune responses that are effective against unmodified glioma cells.

Armed with this information, we were able to proceed with efforts to genetically modify human glioma cells to increase their immunogenicity. Based on our studies (above) and on the reports of others using immunogene therapy in non-glioma animal tumor models (6, 8, 33), we chose to focus on transferring the pro-inflammatory genes B7-2, GM-CSF, and IL-12. Through reverse transcriptase-PCR cloning, we amplified the cDNA's for these genes from human lymphocyte or (for IL-12) keratinocyte RNA and ligated them into a previously constructed retroviral gene therapy vector, pLSN (20). As outlined in chapter 5, we demonstrated that these vectors can efficiently transfer these genes to human brain tumor cells. More importantly, irradiation with 200 Gy (necessary to prevent cell division in genetically altered tumor cell vaccines and, thus, prevent secondary tumor growth at vaccination sites) did not alter expression of the retrovirally transferred genes. Most cells remained viable for 1 to 3 days post-radiation, but this dropped off quickly. Radiation completely blocked clonogenic potential, suggesting that cell division was completely inhibited. Transferring B7-2, GM-CSF, or IL-12 to human brain tumor cultures with our retroviral vectors did not alter their *in vitro* growth rates. These data helped to establish the feasibility of vaccination with genetically altered autologous glioma cells and clarified some basic safety issues.

Finally, we wished to examine immunogene therapy's biologic effects. As outlined in chapter 6, we developed a new human glioma xenograft model in severe combined immunodeficient (SCID) mice reconstituted with allogeneic human lymphocytes. In this system, vaccination with irradiated glioma cells transduced with B7-2 and GM-CSF completely inhibited the growth of pre-established subcutaneous human gliomas. These data demonstrated the potential efficacy of glioma immunogene therapy and, along with pre-clinical data from other groups (9, 21, 22, 31), has set the stage for phase I clinical trials.

Future Directions Arising Directly From This Thesis

In this thesis, the clinical problem posed by human gliomas has been outlined, basic scientific studies regarding human glioma culture techniques and immunobiology have been undertaken, and transitional research studies setting the stage for a phase I glioma immunogene therapy trial have been completed. The natural next step is to initiate such a trial. This, in fact, has occurred. Based largely on the work in this thesis and similar work regarding malignant melanomas (Solano *et al.*, unpublished), we have recently received approval from the federal Health Protection Branch to proceed with a phase I pilot study of vaccination with irradiated autologous B7-2/GM-CSF-transduced tumor cells in the treatment of recurrent gliomas and medically-refractory melanomas. The protocol for this trial is included in this thesis as Appendix 1. This is the only the second gene therapy trial to take place in Canada and the first in Western Canada. It is also the first immunogene therapy trial initiated worldwide utilizing a two gene model.

In addition to a clinical trial, this work leads naturally to a number of other investigations. Several studies intimately related to the work described here are either underway or will be initiated in the near future. For example, there are two major drawbacks of the SCID mouse model (and the data generated from it) described in Chapter 6: the use of allogeneic lymphocytes to reconstitute the mice and the use of a subcutaneous model for an intracranial tumor. We are currently attempting to address these drawbacks. We have developed an *in vitro* immunogene therapy model using early passage human glioma cells and peripheral blood mononuclear cells derived from the same patients. This autologous system has yielded some intriguing preliminary results suggesting that combinations of wild type and B7-2/GM-CSF-transduced glioma cells synergize to stimulate more anti-glioma cytotoxic T cells than B7-2/GM-CSF-transduced glioma cells alone (Parney *et al.*, unpublished). We are currently working to confirm this finding.

In addition to developing an autologous *in vitro* model, we are attempting to improve the SCID mouse model in two important aspects. First, we plan to initiate an intracranial version of the model. Second, we wish to avoid using allogeneic lymphocytes to reconstitute the mice. Pilot studies indicated that reconstituting the mice with patients' lymphocytes and using xenografts from the same patients' tumors was not practical due to the need to withdraw large volumes of blood from sick glioma patients and the unreliable tumorigenicity of many early passage human glioma cultures (Parney *et al.*, unpublished). Therefore, we hope to set up a syngeneic model using lymphocytes from healthy donors to reconstitute SCID/bg mice with MHC-matched human glioma xenografts.

While we have suggested that human glioma cells transduced with IL-12 might be effective at stimulating anti-glioma immunity and demonstrated that we are able to efficiently transduce human glioma cells with IL-12, we have not presented any data demonstrating the efficacy of IL-12-transduced glioma cell vaccines. Clearly, we need to evaluate IL-12-based glioma immunogene therapy directly. Studies are underway using both the autologous *in vitro* model discussed above and the SCID/bg model outlined in Chapter 6. Preliminary results suggest that IL-12-transduced glioma cells do stimulate changes in anti-tumor immunity, but it is not yet clear whether these changes are beneficial (Parney *et al.*, unpublished).

In addition to the studies underway or proposed that proceed directly from the work in this thesis, it should also be recognized that immunogene therapy is potentially applicable to a wide variety of tumors in addition to gliomas. Indeed, many initial studies examining cancer immunogene therapy concentrated on melanomas, sarcomas, and adenocarcinomas (8, 12, 21, 33). It follows, therefore, that the immunogene therapy strategies outlined for gliomas in this thesis may be applicable to other types of tumors. Our laboratory is already exploring this possibility in melanomas (Solano *et al.*, unpublished) and may be able to do the same for other tumors in the future.

Other Potential Future Directions

As with many scientific investigations, the basic immunobiology studies outlined in Chapters 3 and 4 raise several new questions. For example, in Chapter 4, it was noted that human glioma cells induce apoptosis in a murine lymphoma cell line in a Fas-independent fashion when they are co-cultured. This raises two questions. First, what mediates this apoptosis? The apoptotic time course (very rapid) suggests that pre-synthesized cellular components are responsible. It is possible that other members of the TNF-α / TNF-α-receptor superfamilies are responsible, as binding between many members of these families is known to induce apoptosis (16). Alternately, other known or unknown factors may be the culprits. A second, perhaps more important, question is also raised by these findings. Do human glioma cells induce similar Fas-independent apoptosis in human lymphocytes? If so, this may be a further pathway for glioma-induced immunosuppression.

Several studies could be initiated as an initial attempt to answer these questions. First, the ability of antibodies to known apoptosis-inducing ligands (eg. TNF-α, Fas-ligand, GM2) to block human glioma-induced apoptosis in the murine lymphoma cell line BW5147. Second, it can be ascertained whether the same human glioma cell lines that induce apoptosis in BW5147 cells also induce apoptosis in co-cultured human peripheral blood mononuclear cells. If so, the abilities of the same antibodies discussed above to block this apoptosis can be assessed.

In our studies to date, we have examined expression of several important immunologic mediators by human glioma cells. These studies are by no means exhaustive. Many factors, known and unknown, may be important in glioma immunobiology and will require further study. For example, CD40 / CD40-ligand interactions have recently attracted a great deal of attention in basic immunology studies (3, 11, 19, 23). It appears that recently activated T_H cells express CD40-ligand that binds to CD40 expressed by APC's. This activates APC's, resulting in increased class I and II MHC, B7-1, B7-2, and IL-12 expression which further drives immune responses. It has recently been reported that some human tumors (melanomas, epithelial tumors, renal cell carcinoma) express CD40 (14, 17, 25-27). CD40 and / or CD40-ligand expression could have important implications for tumor immunology, and has not been examined in gliomas previously. With our glioma cell culture techniques (see Chapter 2) and frozen glioma tissue bank, we have many tools at our disposal to determine CD40 and CD40-ligand (or other immunologic factors) expression in human gliomas.

New information about glioma immunology may suggest new therapeutic options (particularly for immunogene therapy) that could be evaluated using our SCID mouse and autologous *in vitro* models. For example again, CD40-ligand may be a very attractive target for immunogene therapy. Glioma cells that transduced with CD40-ligand could be immunostimulatory in a number of ways. First, they may stimulate nearby professional APC's such as dendritic cells (if administered subcutaneously) or microglia (if administered intracerebrally) to upregulate MHC molecules, B7 molecules, and IL-12. This alone may very effectively stimulate anti-glioma immune responses. In addition, if gliomas themselves express CD40, CD40-ligand transduction may result in autocrine stimulation leading to increased MHC, B7, and IL-12 expression (in effect, converting glioma cells to "professional" APC's). If this was the case, however, it would be vitally important to determine if CD40-ligand transduction also stimulated glioma growth.

Finally, in addition to new basic immunologic studies and new targets for immunogene therapy, our studies suggest the need for improvements in gene transfer technologies. Retroviral gene transfer is labor intensive, takes several months, and requires robust growth of primary tumor cultures. As a result, it is often impossible to produce a vaccine for a given patient in a timely fashion. Simpler and more universally applicable gene transfer techniques are more desirable. In addition, genetically engineered viruses have the potential to mutate and become replication competent. Although this has yet to occur in any clinical trial involving viral gene transfer vectors and extensive safety tests are required in most jurisdictions to prevent this, such mutations are theoretically possible and could have unknown consequences. Therefore, developing non-viral gene transfer techniques would also be desirable.

Many non-viral gene transfer techniques have already been developed, including calcium phosphate co-precipitation, electroporation, liposomal transfer (with or without specific ligand targeting), naked DNA injection, and biolistic (gene gun) gene transfer (7). While all of these approaches are potentially useful, some are more attractive than others. Calcium phosphate co-precipitation and electroporation are commonly used laboratory techniques for gene transfer. However, their relative inefficiency and potential mutagenesis have limited their clinical application (7). Liposomal transfer and naked DNA injection are two techniques that have been widely investigated for cancer gene therapy (13, 24, 29). However, pilot studies in our laboratory indicate that neither technique is reliable or efficient for transferring reporter genes to human glioma cells *in vitro* or *in situ* (Parney *et al.*, unpublished).

In contrast to other non-viral gene transfer techniques, biolistics have been extremely efficient at transferring genes to human glioma and melanoma cells *in vitro* in our hands (Gainer *et al.*, unpublished). In biolistic gene transfer, fine (1.6 um) gold particles are coated with plasmid DNA for eukaryotic expression vectors and "fired" at tumor cells using a compressed gas jet (30). This "gene gun" has several advantages compared to retrovirus-mediated gene transfer. It does not require cell division for gene transfer and does not require cell selection to generate a highly expressing population. As a result, it requires much less time in tissue culture than retroviral gene transfer. Biolistic gene transfer does not involve infectious or potentially mutagenic vectors. Because of these advantages, gene gun-mediated gene transfer is considerably safer than retroviral gene transfer. This has further advantages because it simplifies the process for creating immunogene therapy vaccines as many time consuming and expensive safety testing procedures (for replication competent virus, etc) can be eliminated. Finally, biolistics allows simple transfer of multiple genes if gold particles are coated with multiple plasmids. This obviates the need to create a single complex gene therapy vector encoding multiple genes. Like all non-viral gene transfer techniques, biolistic gene transfer is episomal (genes are not integrated into chromosomal DNA). As a result, expression is transient. However, this is more than adequate for immunogene therapy vaccines. Thus, biolistics appears to be a safe, simple, and speedy alternative to retrovirus-mediated gene transfer for glioma immunogene therapy and is actively being pursued by our laboratory.

Summary

This thesis outlines several experimental studies in human glioma immunology and immunogene therapy. An efficient technique for culturing human glioma cells has been developed. Using this technique, several studies have been undertaken that indicate that human glioma cells are potentially sensitive to cell-mediated immune responses but express several factors that inhibit the activation of such responses. Using genetically engineered retroviruses, genes encoding pro-inflammatory genes (B7-2, GM-CSF, IL-12) have been transferred to cultured human brain tumor cells. In a novel humanized SCID mouse model, vaccination with irradiated human glioma cells expressing B7-2 and GM-CSF markedly inhibited pre-established human gliomas.

Several projects have been initiated that are direct extensions from the studies in this thesis. First, a phase I clinical trial of vaccination with irradiated autologous tumor cells expressing B7-2 and GM-CSF in the treatment of recurrent gliomas has been recently approved by the federal Health Protection Branch and will be initiated shortly. An autologous *in vitro* model of human glioma immunotherapy has been established and is being used to evaluate IL-12 immunogene therapy. Improvements to the humanized SCID mouse model (intracranial tumor and syngeneic lymphocytes) are also being pursued. In addition, much of the work in this thesis is currently being replicated with human melanomas.

A number of other potential directions have been suggested by this thesis. Many aspects of glioma immunology remain to be fully elucidated. Further information about basic glioma immunology may help focus glioma immunogene therapy efforts on more potent immunostimulatory genes. For example, establishing CD40 and CD40-ligand expression patterns by human gliomas may be an important first step toward exploiting this system to stimulate anti-glioma immunity. In addition, the work in this thesis has underlined the need to develop more efficient non-viral methods for gene transfer in order to increase the safety and universal applicability of glioma immunogene therapy. Initial work in our laboratory suggests that biolistic gene transfer may be one such technique.

Gliomas are complex tumors that generally have a dismal prognosis despite maximal efforts with conventional therapy. This has led to efforts to find novel therapies with greater efficacy. Immunogene therapy is one such promising modality. However, glioma immunology is complicated and incompletely understood. This thesis represents an effort to further define human glioma immunobiology and establish gene therapy techniques to effectively stimulate anti-glioma immune responses. Although the results to date are very exciting, they must be tempered with the knowledge that gliomas are highly malignant and highly resistant to all forms of therapy. It can be anticipated that establishing effective clinical immunogene therapies for gliomas will not be easy. While this thesis and other similar studies may represent the first steps towards this goal, it is clear that a great deal of work remains to be done.

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Appendix 1

Clinical Protocol: pLSNBG9 / Autologous Tumor Cell Vaccines in the Treatment of Recurrent Gliomas and Medically Refractory Melanomas

1.0 INTRODUCTION/BACKGROUND

1.1 Summary of proposed combination immunogene therapy regimen

Malignant gliomas are the most common primary neoplasms of the central nervous system. Despite aggressive multimodality therapy, they continue to have a dismal prognosis. Mean survival for patients with glioblastoma multiforme tumors is less than one year from time of diagnosis. Similarly, prognosis in patients with metastatic melanoma refractory to standard therapy face a dismal prognosis. These ineffective treaments have stimulated investigation of novel therapeutic modalities including gene therapy.

This study is a pilot clinical trial of combination immunogene therapy using genes encoding the T cell costimulatory molecule B7-2 and the proinflammatory cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) in the treatment of five patients with recurrent malignant gliomas and five patients with medically refractory metastatic melanomas. Fresh tumor samples will be obtained at surgery. From these, tumor cells will be grown in tissue culture. Cultured cells will be genetically modified *in vitro* to express B7-2 and GM-CSF by retroviral transduction. Following cell selection *in vitro* and confirmation of therapeutic gene expression, cells will be expanded, aliquoted, and frozen in liquid nitrogen for storage. When (or if) a patient presents with tumor recurrence (for gliomas) or progression despite standard therapy (for melanomas), an aliquot of B7-2/GM-CSF-transduced tumor cells will be thawed and grown in tissue culture. After cells from this aliquot undergo safety testing (for sterility and freedom from replication-competent retrovirus) by an independent certified laboratory, further aliquots from this lot will be thawed as required for clinical purposes. Prior to clinical use, these cells will be irradiated (200 Gys) to prevent further cell division and growth. Irradiated, B7-2/GM-CSF-transduced tumor cells will be given to glioma and melanoma patients as three separate subcutaneous vaccines given at two week intervals. Patients will be followed closely for any signs of toxicity related to therapy as well as systemic anti-tumor immune responses. Patients' clinical course after induction of therapy, including quality of life issues, will be scrutinized and documented.

1.2 Prior Submissions

None

1.3 Objectives

The primary objective is examining the safety of vaccination with irradiated autologous tumor cells genetically modified to express B7-2 and GM-CSF in the treatment of recurrent gliomas and medically refractory melanomas. Secondary objectives include assessing systemic anti-tumor immune responses and clinical course after therapy.

1.4 Timetable

The projected time for enrollment of new cases is 6 months. Individual patients will be followed according to the study schedule for an extended period of time. Initial laboratory and clinical response results (including toxicity) will be available within one year of enrollment. Therefore, the projected time for completion of this pilot study is 18 months.

1.5 Characteristics of Malignant Gliomas

Malignant gliomas such as glioblastoma multiforme are the most common primary central nervous system (CNS) neoplasms. They affect patients of all ages, but most commonly present in the mid fourth to sixth decades of life. To date, no therapeutic regime can be considered curative and the mean survival for glioblastoma patients from time of initial diagnosis is approximately one year despite aggressive multi-modulatory therapy (1, 2). Death is usually due to aggressive local recurrence and concomitant peritumoral cerebral edema. Metastasis outside the CNS is rare. Survival from time of recurrence is usually less than 4 months (3). This dismal prognosis has not improved despite advances in diagnosis, surgical technique, radiation therapy, chemotherapy, and, more recently, adoptive immunotherapy. However, recent advances concerning the molecular biology of these tumors (4-6) have led to study of novel molecular treatment methods, including gene therapy (3, 7, 8). It is hoped that these new modalities will eventually lead to improved survival for malignant glioma patients.

1.6 Characteristics of Malignant Melanoma

The incidence of malignant melanoma is rising faster than any other cancer in Canada. The annual increase in rate is 6.8% in men and 4.8% in women. It is estimated that 1 in every 200 Canadians will develop melanoma at some point in their lifetime (9). In contrast to other forms of skin cancers which are usually curable with surgery, malignant melanoma is often fatal due to its aggressive nature and tendency to early spread to distant organs. The treatment of primary melanoma is surgical while chemotherapy is indicated in patients with metastatic disease. Unfortunately, clinical response to chemotherapy is only approximately 25% and 5 year survival is approximately 5%. Recent clinical trials utilizing interferon α2a and α2b have been instituted although toxic side effects remain a significant limitation of these therapies (10, 11).

1.7 Cancer gene therapy

Gene therapy is the introduction of specific genes into cells for the purpose of treating disease. Initial clinical gene therapy trials began in 1990 for the treatment of severe combined immunodeficiency (12, 13). This seminal study has stimulated investigation of gene therapy strategies in the treatment of many other diseases, including cancer.

A number of cancer gene therapy strategies have been identified. These include:

i) suicide gene transfer therapy: transfer of genes which either kill tumor cells directly or increase their susceptibility therapeutic agents (e.g. HSV-tk gene / ganciclovir therapy) (14-18).

ii) inhibition of cell proliferation: replacement of a defective or absent tumor suppressor gene (6, 19).

iii) immunogene therapy: transfer genes that encode immunostimulatory protiens to tumor cells (20-26).

With the exception of immunogene therapy, all other gene therapy approaches require efficient *in situ* gene transfer and expression in target cells for successful anti-tumor response. Unfortunately, adequate biodistribution of gene transfer into tumors remains an inherent obstacle for successful anti-tumor effects. For example, transduction rates for HSV-tk gene transfer to glioblastoma tumors in animal models vary widely from 10% to 70% (15, 16, 27, 28). Although stable enhancer and promoter combinations that promote optimal expression of the therapeutic genes continue to be identified (29-33), incomplete gene transfer still limits the efficacy of these approaches.

Difficulties encountered in *in vivo* cancer gene therapy due to poor gene transfer efficiency may be overcome by shifting to *ex vivo* gene transfer strategies such as immunogene therapy. This strategy is based on vaccination with irradiated tumor cells genetically modified *in vitro* to increase their immunogenicity. This is an attractive alternative as gene transfer and expression into early passage tumor cultures *in vitro* can be performed simply and efficiently (21). Furthermore, this method avoids the problem of subtotal gene expression as transduced cells can be selected *in vitro* prior to immunization via co-transduced antibiotic resistance genes.

1.8 Cancer Immunotherapy

Cancer immunotherapy refers to stimulating host immune responses to combat cancer. Passive cancer immunotherapy involves the systemic administration of immunoreactive substances such as cytokines or immunoglobulins that may have anti-neoplastic effects. This approach does not involve stimulation of the patient's own immune system *per se*. Active cancer immunotherapy involves vaccination with autologous tumor cells to stimulate the patient's own immune response against the tumor(s). Both passive and active immunotherapy strategies have been studied in glioblastoma patients previously, but unfortunately were not associated with improved clinical responses (34).

Failure of previous cancer immunotherapeutic strategies may reflect the low and nonspecific antigenicity of malignant cells, the secretion of immunosuppressive factors (such as transforming growth factor ß2, prostaglandin E2, interleukin-10) by tumor cells, and the unrelenting outgrowth of immunologically variant neoplastic cells invariably seen in refractory cancers (35-41). In addition, defective T cell function has hindered previous attempts at cancer immunotherapy.

These factors can potentially be overcome with sufficient stimulation of antigen-presenting cells (APCs) and support from appropriate cytokines (e.g. IL-2, GM-CSF, IL-12, etc.). For example, dendritic cells are highly effective APCs physiologically for induction of primary immune responses. These cells are potentially exploitable for for delivery of tumor-specific antigens in cancer immunotherapy (42). Alternatively, tumor cells may be genetically engineered to express molecules (T cell costimulators, proinflammatory cytokines) normally expressed on highly efficient APCs such as dendritic cells. This latter strategy, known as immunogene therapy, may be advantageous as it includes a ready source of tumor antigens.

1.9 Cancer immunogene therapy

Cancer immunogene therapy is a form of active immunotherapy in which patients are vaccinated with irradiated, autologous tumor cells that have been genetically modified *in vitro* to increase their immunogenicity. This has approach has inhibited local and systemic tumor growth in several animal tumor models (21, 43, 44) and is now in Phase I clinical trial in the treatment of renal cell carcinoma (45).

Immunogene therapy for gliomas has been reported in a small number of studies involving animal models (26, 46, 47) and in one clinical case report (23). These studies reported tumor growth inhibition after vaccination with autologous glioblastoma cells transduced with either interleukin-2 (IL-2), interleukin-4 (IL-4), or transforming growth factor β2 antisense gene. Melanoma immunogene therapy has been investigated using both subcutaneous and intracranial animal tumor models. In particular, several studies have examined the role of B7 T cell costimulatory molecules in stimulating anti-melanoma immune responses (48-51). In addition, a recent study by Sampson, *et al.* (20) has demonstrated that subcutaneous vaccine of mice with irradiated, autologous melanoma cells expressing murine GM-CSF inhibits growth of pre-existing intracranial melanomas. Immunogene therapy strategies for melanoma are now in clinical trial (52, 53) and one case of a patient with metastatic melanoma treated with irradiated autologous GM-CSF-secreting tumors cells has been reported (54).

In our own laboratory, we have recently completed studies examining immunogene therapy for human glioblastoma multiforme using tumor cells transduced with human B7-2 and GM-CSF genes in SCID mice reconstituted with allogeneic human lymphocytes (55). We demonstrated efficient B7-2 and GM-CSF gene transfer to human glioblastoma cells *in vitro* via retroviral vectors developed in our laboratory. Growth of tumors expressing GM-CSF alone was moderately inhibited. Growth inhibition was marked for tumors expressing B7-2. Vaccination with glioblastoma cells transduced with both B7-2 and GM-CSF genes markedly inhibited growth of wild type tumors (including pre-existing tumors) at distant sites. Similar work is under way for human melanomas (Solano, *et al.*, unpublished).

1.10 Immunotherapuetic genes: cytokines and costimulatory molecules

Many immunomodulatory genes are potentially useful and may be necessary for overcoming tumor immunosuppression. These include genes encoding cytokines, major histocompatibility complex molecules, or T cell costimulatory molecules (43, 56). An ideal tumor vaccine should coexpress a combination of costimulators, cytokines, and chemoattractive adjuvants. Our study has focused on genes from two distinct immunomodulatory pathways: the therapeutic cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) and the T-cell costimulatory molecule B7-2.

GM-CSF stimulates growth and differentiation of granulocytes, monocytes/macrophages, and other antigen presenting cells. From an immunotherapeutic perspective, this cytokine has been shown to promote maturation of cutaneous dendritic cells, enhance antigen presentation by APCs, and stimulate B7 costimulator molecule production by tumor cells (42). GM-CSF has recently come into widespread clinical use as a treatment of neutropenia due to its hematopoietic effects (57-59). Its importance in tumor immunogene therapy was demonstrated by Dranoff, *et al.*. (43) who showed that vaccination with irradiated GM-CSF-transduced tumor cells produced specific and marked growth inhibition of wild type tumor challenges in mouse models of adenocarcinoma and melanoma. Of a panel of 10 cytokine genes tested, tumor growth inhibition was greatest for GM-CSF.

B7-2 is one of a family (B7-1, B7-2, and B7-3) of macrophage/APC cell surface molecules that have recently been identified as costimulatory molecules necessary for T-cell activation (via CD28) in conjunction with antigen presentation in the context of a major histocompatibility complex molecule (60-64). The absence of costimulatory molecules on tumor cells may contribute to their avoidance of immune surveillance (65). The specific roles of the various costimulatory molecules are yet to be clearly defined. It has been suggested that B7-1 expression promotes differentiation of intermediate T_H cell precursors into T_H1 effector cells (cellular immune responses) while B7-2 expression leads to T_H2 differentiation (humoral immune responses) (66, 67). However, both B7-1 and B7-2 have been shown to promote cell mediated immune responses in animal models (68-70). More recently, the effectiveness of B7-1 expression in promoting tumor rejection has been questioned (71). Furthermore, T cells must receive costimulatory signals from antigen-presenting cells (APC's) within the first 12 hours of T cell receptor stimulation for maximal IL-2 production (72). Therefore, the rapid induction of B7-2 (not B7-1) on APC's after antigen stimulation suggests that B7-2 is the preferable costimulatory molecule to promote antitumor cell-mediated immune responses (65). Given that CD28 and B7-2 exhibit similar temporal patterns of increased expression after activation of naive T and B cells, it now appears that B7-2 is the principal ligand for CD28 T cell co-receptor (73).

1.11 Combination *vs.* single gene immunotherapy

We have focused on the novel combination of GM-CSF and B7-2 for immunogene therapy because these two immune modulators activate distinctly different pathways for immunostimulation. As its name suggests, glioblastoma multiforme is a highly heterogeneous tumor made up of many cellular phenotypes. It is probable that the anti-tumor immune response obtained after transfer of a single gene will vary from tumor to tumor (or different cell populations within a single tumor). Therefore, a "cocktail" of genes stimulating different immunoreactive pathways is more likely to induce an anti-tumor immune response than any single gene. Combination cytokine and T cell costimulatory molecule immunogene therapy has been shown to be synergistic (for interleukin-2 and B7-1 in the treatment of murine fibrosarcoma) in at least one previous report (74).

1.12 The Role of the "Alarm Signal"

For glioma patients who are undergoing further resections of recurrent tumors, we propose to initiate subcutaneous vaccinations within 3 days of tumor debulking to capture the "alarm signal" (75, 76) that may be required for costimulator activation on APCs (i.e. dendritic cells and microglia) and enhancement of activated macrophage and T cell traffic across the blood-brain barrier. The precise nature of the alarm signal remains to be determined. It has been suggested that alarm or danger signals are mediated through CD40-ligand binding CD40-receptor on antigen presenting cells, but this is unknown at the present time (77). Regardless of the precise molecular nature of this signal, tissue disruption appears to be important in causing its activation(75, 76). Therefore, we plan to time the first vaccination within the 3 days of surgery for glioma patients undergoing repeat resection. Because patients with refractory melanomas will not be undergoing further surgery in this protocol, timing of their first vaccination is not as critical.

2.0 METHODOLOGY

2.1 Tumor harvesting, processing, and culturing

One to three grams of tumor are harvested when patients originally present and undergo surgery. These biopsies are obtained under sterile operating room conditions. Samples are dissected to remove necrotic debris and connective tissue and minced into 1-2 mm pieces. For gliomas, partial enzymatic digestion is carried out by incubating in Hank's Buffered Saline Solution (HBSS) containing 0.025% collagenase IV, 0.04% DNase and 0.05% pronase for 30 minutes at 37°C and a further 30 minutes at 4°C. For melanomas, enzymatic digestion takes place by incubating minced tumor in a solution containing 0.20% collagenase II and 0.01% hyaluronidase for 3 hours at room temperature (with stirring). The resulting suspensions are filtered through a tissue culture sieve and layered onto a density gradient medium (Ficoll) before centrifuging at 400xg for 30 minutes at room temperature. The cells at the density gradient interface are removed and washed twice with HBSS. Glioma cells are seeded in Dulbecco's Modified Eagle's Medium/F12 (DMEM/Fl2) medium supplemented with 10% Fetal Bovine Serum (FBS) while melanoma cells are grown in similarly supplemented RPMI1640 media. All cultures are grown at 37°C, 5% CO_2. Tumor cells are grown until sufficient numbers are present to allow retroviral gene transfer and selection (see below). All tumor processing and culturing will be done in our own laboratory. Our methods for establishing human brain tumor cultures have been previously reported (78).

2.2 Retrovirus-mediated gene transfer into early passage tumor cultures

The retroviral vector utilized in this study (pLSNBG9) is derived from Maloney leukemia virus (MLV). It is similar to vectors employed by a number of investigators in *in vitro* and *in vivo* studies including approved investigations with human subjects (79). Generation of the retroviral backbone (pLSN) has been previously reported (33). pLSNBG9 is a bicistronic vector contains human B7-2 and GM-CSF cDNAs under the control of the MLV LTR. The two therapeutic genes are linked by an internal ribosome entry site (IRES) derived from the murine encephalomyocarditis virus genome (Novagen). This vector is missing the gag, pol, and env genes necessary for viral packaging and is thus rendered replication incompetent. These gene products are provided in *trans* by PA317, a retroviral packaging cell line (80, 81).

Briefly, the transduction protocol can be described as follows. Virus stock is thawed from -80°C at 37°C. Polybrene is added to the thawed virus solution at a final concentration of 4 μg/ml. Polybrene is a polycationic molecule that facilitates retroviral infection, presumably by neutralizing negative charges present on the surface of cells and virions to provide better binding(81). Culture media is added to the virus solution to bring the final volume to 1.5 ml. Logarithmic growth phase tumor cells in T25 flasks are incubated in the virus supernatant at 37°C, 5% CO_2 for 3 hours. The same volume of media and polybrene without virus is used as control. After 3 hours, a further 2.5ml of culture media is added to each flask and they are incubated overnight. The media is removed the next morning, and the cells are exposed to fresh virus as before. This is repeated for a third time on the following day. On day 4, 250 μg/ml of the neomycin analogue G418 is added to culture media. Media is changed every two to three days until complete selection had taken place (i.e. all cells in the control flasks are dead). After selection, cells are cultured in growth media containing reduced concentrations of G418 and allowed to grow to confluence.

The methods utilized for transfer of therapeutic genes are identical to those employed in previously approved human gene transfer studies in which autologous tumor cells were modified by retroviral gene mediated transduction and administered to patients harbouring renal cell cancers (82). Thus far, no infectious retroviruses have been identified in these patients and no significant side effects due to gene transfer have been noted. In addition, a phase I/II immunogene therapy protocol for the treatment of hormone-refractory prostatic carcinoma has been approved by the Recombinant Advisory Committee (RAC) of the National Institutes of Health of the U. S. In this study, surgically excised autologous prostate tumor cells are genetically modified to express GM-CSF for the systemic stimulation of antitumor cytotoxic T-lymphocytes (Human Gene Transfer Protocol #9408-082).

2.3 Gene expression analysis

Transduced tumor cells are split into a new T25 culture flask at a density of 1×10^6 cells. The amount of GM- CSF secreted into culture medium is determined by ELISA (Quantikine, R&D Systems) 24 hr later. B7-2 expression is evaluated in a non-quantitative fashion by flow cytometry using a fluorescien-conjugated monoclonal antibody specific for human B7-2 antigen (Ancell).

2.4 Tumor cryopreservation and thawing

Tumor cells that have been transduced with GM-CSF and B7-2 genes, selected, and expanded will be cryopreserved for future use. Briefly, cells are aliquoted in small volumes into cryopreservation tubes at 1×10^6 cells/tube. Total volume is made up to 0.5 ml with a mixture of DMEM/F12, FBS, and DMSO to make a final concentration of 10% FBS and 25% DMSO. Cells are placed in an insulated styrofoam rack and placed at -80°C for 24 hours, then placed in liquid nitrogen for long term storage. When cells are needed, they are thawed from liquid nitrogen at 37°C and washed twice in 0.9% sodium chloride solution. They are resuspended in 1 ml of 0.9% sodium chloride solution and taken immediately for irradiation.

2.5 Irradiation of tumor cells

Transduced tumor cells will be irradiated prior to their reinjection to patients to prevent further cell division. This eliminates the possibility of tumor growth at vaccination sites due to introduction of new neoplastic cells. Cells will be treated with 200 Gy in the Gammacell 1000 irradiator available in the Red Cross Building adjacent to the W.C. Mackenzie Health Sciences Center at the University of Alberta.

2.6 Safety testing

Tumor processing and gene transfer will occur in our own facilities at the University of Alberta. This, of course, is contingent on adherance to strict safety testing in concordance with the "points to consider" for retroviral gene therapy vectors in clinical trials recently published by the Food and Drug Administration of the United States (see Appendix 8). Aliquots of cells to be used as vaccines in this trial will need to pass rigorous safety testing prior to their use in clinical applications. Our own safety testing will include observation for bacterias and fungal contamination and mycoplasma testing. If cells pass all safety testing in our own laboratory, an culture supernatants and cell lysates (representing 10% of the clinical lot) and two aliquots of cryopreserved cells (representing a further 20% of the clinical lot) will be forwarded to a certified independent laboratory (MA BioServices) for further safety testing. This will include direct inocculation tests for microbial contamination, mycoplasma assays, and tests for replication-competent retrovirus (see Appendix 5 of this volume for further details). Safety testing at MA BioServices will be performed at Good Laboratory Practices standards. If cells pass the independent safety assessment, then aliquots from the same lot will be thawed and used for vaccination as appropriate. In addition, patients' serum will be analyzed in our laboratory before and after vaccination for replication-competent retrovirus via the S+L- assay (83).

2.7 Administration of biological agents

Patients will receive three subcutaneous injections in total. Each vaccination will consist of 2 x 10^6 irradiated, autologous tumor cells modified to express B7-2 and GM-CSF. Injections will be given on alternating lumbar flank regions. They will be marked immediately above by a small India Ink tattoo to allow accurate localization later in the event that local reaction is not apparent. Patients will receive 1.0 ml of vaccine injected subcutaneously at each site using a 1 ml syringe and a 23 gauge needle. All vaccinations will be prepared by resuspending cells in sterile 0.9% sodium chloride solution. For patients with recurrent gliomas undergoing further debulking, the first injection will be on the first or second post-operative day. For all other patients, the timing of the first injection is not as critical as these patients are not undergoing any further surgical resection of their tumors. All patients will receive a second and third vaccination 14 and 28 days (respectively) after the first vaccination.

With the exception of the first vaccination in patients with recurrent gliomas (who will likely be post-operative inpatients at the time), all subjects will be treated as outpatients. Vital signs will be monitored prior to immunization and every half hour for 3 hours after the subcutaneous injections. Patients will be examined every hour for 3 hours for inflammation at the injection site and for evidence of rash, wheezing, or edema. Provided there are no contraindications, subjects will be discharged 3 hours after treatment. Should significant reactions occur, the patients will be hospitalized for constant monitoring.

2.8 Dose escalation

A standard practice for dose escalation in phase 1 clinical trials for cancer chemotherapy is to use an initial dosage of 10% of an estimated LD50. This dose is then increased using a modified Fibonacci series (84). This approach presents significant difficulties in this study. The B7-2 molecule is a cell surface antigen that is normally present on antigen presenting cells like tissue macrophages, dendritic cells, and activated B cells. It is difficult to quantify the amount of B7-2 present in a given sample; only its presence or absence on the surface of cells can be reliably ascertained. Consequently, there is no way to estimate an LD50 for B7-2.

GM-CSF production can be quantified. In trials using GM-CSF to treat chemotherapy induced neutropenia, treatment limiting severe toxicities have been observed at doses > 20 mg/kg/day (57). Based on this, one could estimate an LD50 for GM-CSF at 30 mg/kg/day. Using the pLSNBG9 vector, we have achieved average GM-CSF expression levels of approximately 5 ng/10^6 cells/day in cultured glioma or melanoma cells. In a 70 kg individual receiving a vaccination of 1 x 10^6 cells, this translates to a GM-CSF dose of approximately 70 pg/kg/day. If we were to take 10% of predicted LD50 (3 mg/kg/day) as a starting dose, it would still be more than 4000 times greater than the total dose of GM-CSF produced if an entire clinical lot (1 x 10^7 cells) was used for a single vaccine (0.7 ng/kg/day). This starting dose would be more than 10 000 times the dose used in previous clinical trials of GM-CSF cancer immunogene therapy (45, 54).

Because of these unique aspects to this study, it is impossible to design a standard dose escalation protocol starting at a dose estimated to be 10% of the LD50. In addition, we are unable to produce large amounts of pLSNBG9 / autologous tumor cell vaccines for any given patient due to the inherent difficulties involved in primary tumor cell culture. This limits our average clinical lot size to approximately 1 x 10^7 cells, markedly limiting the amount of vaccine available for dose escalation. Furthermore, generation of systemic antitumor immunity is unlikely to be dose related. Rather, it should be related to the ability of the vaccine to promote activation and proliferation of antitumor lymphocytes. With all this in mind, we have chosen an arbitrary and constant cell number for each vaccination and in all patients (1 x 10^6 cells) rather than attempting "dose escalation."

2.9 Overall treatment plans for specific patient subgroups

2.9 a Recurrent glioma patients

As stated previously, tumor samples will be obtained for culturing when patients undergo resection of their gliomas. These cultures will undergo gene transduction, selection, expansion, and safety testing. These patients will meanwhile undergo adjuvant therapy as determined by their oncologist. This typically consists of local radiotherapy. If patients present with recurrent tumor after resection and radiotherapy and if their tumors have been successfully cultured, transduced, and passed all safety testing, they will be eligible for entry into the study. Unfortunately, such tumor recurrence will likely occur within nine months of the initial diagnosis and treatment in the majority of glioblastoma multiforme patients. For patients who consent to treatment, tumor cell aliquots from a lot that has successfully passed all safety testing will be thawed as required for vaccination. Patients presenting with a surgically accessible first recurrence will undergo repeat resection of their tumors where as patients who have already had one or more recurrences may or may not undergo further debulking depending on their clinical status. Therapy will be initiated with the first vaccination on day 2 or 3 post-op for patients undergoing further debulking. The remainder of the therapy will be carried out as described elsewhere.

2.9 b Medically refractory melanoma patients

This group of patients will be identified for possible later entry into the study at the time of their presentation with metastatic melanoma. Tumor samples will be obtained for culture either at the time of original resection or from lymph node or skin lesion biopsy at the time of presentation with metastasis. Patients demonstrating progressive disease despite maximal conventional therapy will be eligible for entry into the trial. As these patients will not be undergoing further surgical resection, timing of the first vaccination is not as critical. Therapy will proceed as described elsewhere.

2.10 Potential toxicity

Local toxicity at the sites of immunizations is not expected to be significantly detrimental to the patient's health. If marked inflammation occurs at the site of injection, topical steroids will be applied, the skin biopsy (see immunologic monitoring below) may be performed earlier, or, in cases of severe inflammation, wider surgical excision may be performed.

Systemic hypersensitivity reactions are unlikely to occur since the injected material is comprised of low amounts of irradiated, minimally modified autologous cells. Anaphylactoid type hypersensitivity reactions are not anticipated. However, should one occur, administration of epinephrine, fluids, steroids and cardiopulmonary support would be instituted as required.

Because B7-2 is normally expressed on many cells, we do not expect significant toxicity related to its use. Any such toxicity would be expected to be exclusively local as it is a cell surface molecule that is not secreted into systemic circulation.

The potential toxicities of GM-CSF secretion have been summarized by Berns *et al.*. in their clinical trial protocol for treating patients with metastatic renal cell carcinoma (82). Although GM-CSF has been reported in isolated cases to promote tumor cell growth in tissue culture for a number of tumor types (85, 86), other studies have failed to show any promotion of tumor cell growth *in vitro* by GM-CSF for a number of other tumor types (87, 88). This tumor promoting effect of GM-CSF has not been seen *in vivo* (89). Indeed, GM-CSF has recently come into widespread use clinically in the treatment of acute neutropenia after myelotoxic cancer therapy (90-92). There has been no documented increase in tumor growth secondary to GM-CSF in these patients.

Systemic GM-CSF administration clinically has been associated with some toxicities, particularly at higher doses (57). At doses of 0.3-10 mg/kg/day, these toxicities include: lethargy, myalgia, bone pain, anorexia, nausea, vomiting, weight change, skin eruptions, flushing, and fever and chills. Doses of >20 mg/kg/day were not well tolerated because of weight gain with fluid retention, pleural and pericardial inflammation, and venous thrombosis. While all of these are potential toxicities in this study, their occurrence secondary to GM-CSF administration is exceedingly unlikely as the dose of GM-CSF administered in this method is more than 10 000 times less (70 pg/kg/day) than the doses described above, it is administered locally (not systemically), and the cells used in vaccination have been irradiated (preventing cell division and subsequent increase in GM-CSF dose).

Theoretical toxicities related to the use of retrovirus are unlikely as the vector has been rendered replication deficient by the deletion of the viral gag, pol and env genes. Safety testing for replication-competent retrovirus as outlined previously should limit this potential toxicity.

The neoR gene transferred into the genetically modified tumor cells or fibroblasts inactivates neomycin intracellularly. Systemic ablation of neomycin treatment in patients receiving these retroviral vectors is thus unlikely. In addition, other more commonly used antibiotics of this class such as gentamicin and tobramycin are not affected by the neoR gene.

As the tumor cells used as vaccines will be cultured for some time prior to their use in clinical applications, contamination and subsequent transmission of infection to the recipient is a potential toxicity. This should be minimized by strict adherence to tissue culture technique and to safety testing.

Stimulating a strong anti-tumor immune response by this protocol does have its own potential toxicities. For glioma patients, this could result in increased peritumoral cerebral edema and increased intracranial pressure. For melanoma patients, a strong anti-tumor immune response could result in a tumor lysis syndrome (particularly if the patient has a large tumor bulk). Should these problems arise, appropriate therapy would be instituted.

Finally, immune system stimulation may result in an increased incidence of autoimmune diseases including (but not limited to) rheumatoid arthritis, systemic lupus erythematosis, Crohn's disease, and ulcerative colitis. No such effects have been seen in our own or other animal studies and none have been reported to date in clinical trials. However, this remains a theoretical toxicity.

2.11 Toxicity assessment and reporting

All patients will be closely observed for the first 3 hours after administration of each vaccine at which time they will be discharged if appropriate. They will be contacted by phone daily for two days after injection to inquire about their symptoms and will be asked to return to the clinic if these require further evaluation. Regardless, they will be assessed in the clinic three days after vaccination. For the next 8 weeks, they will be evaluated weekly. Thereafter, they will be followed monthly for 4 months, every 3 months for 1 year, and, finally, yearly. At each of these visits, a pertinent history and physical will be obtained. Patients will have a urinalysis and have blood samples taken for CBC, electrolytes, urea, creatinine, glucose, PTT, INR, total bilirubin, alkaline phosphatase, liver enzymes, calcium, magnesium, amylase, and lipase at each scheduled assessment as outlined above. In addition, patients will have blood drawn for replication-competent retrovirus assays one week after each vaccination.

All patients will be observed for any toxicities. These will be graded according to the National Cancer Institute of Canada Clinical Trials Group (NCIC CTG) expanded common toxicity criteria: 0 (none), 1 (mild), 2 (moderate), 3 (severe), 4 (life-threatening), and 5 (lethal). These toxicities are classified into 11 categories based on body systems and are further divided into several toxicity variables based on common toxicities.

NCIC CTG grade 2 and 3 adverse reactions will be reported in writing within 10 working days to the University of Alberta Research Ethics Board (REB) and the federal Health Protection Branch (HPB). Any grade 4 adverse reaction or patient death while on gene therapy treatment will be reported to the REB and HPB by phone within 24 hrs with a written report to follow within 10 working days.

2.12 Immunologic monitoring

Patients' immunologic reaction to immunogene therapy will be monitored both locally and systemically. Local immune response will be monitored by symptoms and signs of delayed type hypersensitivity (DTH) responses at the vaccination sites. In addition, punch skin biopsies will be performed at the sites of injection 2 weeks after each vaccination (i.e. days 14, 28, and 42). These will be compared to a biopsy taken from normal lumbar flank skin on day 1 (prior to initiation of therapy). Biopsies will be performed under sterile conditions with local anesthetic using a 4-5 mm punch biopsy knife. A single suture may be required for each biopsy site. These biopsies will undergo standard pathologic examination for evidence of tumor cells and inflammation. In addition, immunohistochemical staining for CD45, CD4, CD8, and NK cell markers will be performed. This pathologic examination and immunohistochemical analysis will be performed by Dr. Charles Hao (Neuropathology, Deparment of Pathology, University of Alberta).

Systemic immune responses will be measured using three separate assays. First, serum C-reactiveprotein will be determined from samples drawn at each scheduled visit. This has been previously used as a general measure of inflammatory response in GM-CSF immunogene therapy trials (54). In addition, blood samples (20 ml each) will be obtained on days 0, 7, 21, 35, and 49. These will be used to isolate peripheral blood mononuclear cells (PBMC) by centrifugation on a density (Ficoll) gradient. PBMC will then be further stimulated *in vitro* by coincubation for 5 days with irradiated (200 Gy) autologous tumor cells. These PBMC will be used for two further assays. First, a standard ^{51}Chromium release cytotoxic T lymphocyte (CTL) assay will be performed *vs.* autologous tumor cells. Second, intracellular flow cytometry staining for interferon-γ production after exposure to autologous tumor cells will be performed. The ^{51}Cr release CTL assay is a standard assay for cell mediated immunity. It is advantageous as it gives direct information concerning the ability of stimulated PBMC to kill tumor cells. However, it has a relatively low sensitivity. To overcome this problem, the much more sensitive intracellular interferon-γ flow cytometry will also be used. This assay determines the percentage of PBMC present that produce interferon-γ in response to exposure to autologous tumor cells. Since interferon-γ production is closely associated with T_H1 (cell mediated) immune responses, the number of PBMC producing interferon-γ in response to exposure to autologous tumor can be taken as a measure of cell mediated immunity.

2.13 Assessment of clinical status and quality of life

Although this pilot study is designed primarily to look at issues of safety, it is nevertheless imperative to examine clinical status following treatment. This will give an indication of what, if any, efficacy this treatment may have and may indirectly provide evidence of systemic anti-tumor immunity. Furthermore, it will help establish a framework for potential phase II and III trials of this therapy.

Clinical status will be followed by history, physical, and laboratory parameters as outlined above under Toxicity Assessment and Reporting. Appropriate diagnostic imaging tests (e.g. MRI scans with and without gadolinium enhancement) will be obtained at 8 and 24 weeks, every 3 months for the following year, and yearly thereafter. In addition, quality of life will be assessed via the Functional Assessment of Cancer Therapy (FACT) version 3.0 developed at St. Luke's Medical Center, Chicago, Illinois by Dr. D.F. Cella. Patients will be asked to fill out this form every 4 weeks for the first 22 weeks, every three months for the following year, and yearly thereafter.

Response will be defined as summarized below:

1. *Complete Response* (CR): Disappearance of all measurable or evaluable disease, signs, symptoms and biochemical changes related to the tumor, for > 4 wks, during which time no new lesions may appear. At the time of suspected CR, re-evaluate completely, short of invasive procedures.

2. *Equivocal complete response* (ECR): This rating will be assigned where there is complete response but examinations remain abnormal in such a way as to preclude an unequivocal statement that the tumor has completely disappeared.

3. *Partial response* (PR): This rating will be assigned where there is a reduction by at least 50% of the product of the two longest perpendicular diameters of the lesion or lesions without the appearance of new lesions. A bone response consisting of recalcification of lytic bone metastases will be included as partial response providing there has been no disease progression elsewhere.

4. *Minor response* (MR): This rating will be assigned where there is a reduction of < 50% of the product of the two longest perpendicular diameters of the lesion or lesions without the appearance of new lesions, and either no change in or partial recalcification of all osteolytic lesions.

5. *Objectively stable* (S): This rating will be assigned where there is < 25% increase in the product of the two longest perpendicular diameters of the lesion(s) without the appearance of new lesions.

6. *Progressive disease* (P): This rating will be assigned where there is an increase of 25% in the product of the two longest perpendicular diameters of the lesion(s) or the occurrence of new lesion(s).

3.0 STUDY ADMINISTRATION

3.1 Location

Patients will be treated at the Walter C. Mackenzie Health Sciences Centre at the University of Alberta in Edmonton, Alberta, Canada.

3.2 Number of patients

Five patients with recurrent gliomas and five patients with melanoma refractory to conventional therapy will be enrolled.

3.3 Study parameters

3.3 a. Pretreatment evaluations and inclusion criteria

Patients 18 years or older harboring recurrent gliomas or melanoma refractory to conventional therapy are potentially eligible for enrolment. The following pretreatment evaluations will be undertaken:

1. History and physical examination: these evaluations will include a description and quantification of disease activity.

2. Performance status assessment: the FACT Questionnaire will be completed and Karnofsky score will be assigned.

3. Pretreatment laboratory evaluations: CBC with leukocyte differential, INR, PTT, glucose, urea, creatinine, electrolytes, C-reactive protein, SGOT, SGPT, LDH, alkaline phosphatase, bilirubin, uric acid, calcium, total protein, albumin, amylase and lipase, urinalysis, PCR of peripheral blood leukocytes for neoR and retroviral env genes.

4. Other pretreatment evaluations: chest x-ray, disease appropriate (e.g. head for glioblastoma patients) MRI with and without gadolonium enhancement. These studies must be performed within 2 weeks of initiation of therapy.

Patients must start treatment within two weeks of enrolment. For some patients with recurrent gliomas, the first vaccination will occur on post-operative day 2 or 3 after resection of their recurrent tumor. For patients with melanoma, the timing of their first vaccination is not as critical.

3.3 b. Exclusion criteria

Exclusion criteria include the following: autoimmune disorders, significant heart disease (myocardial infarction within 6 months, unstable angina, congestive heart failure), pregnancy (all women in child bearing age must have negative pregnancy test), anemia (hemoglobin < 9.9 g/dl), neutropenia (total granulocytes < 1999/mm^3), thrombocytopenia (platelets < 100 000/mm^3), renal failure (creatinine > 120 µmol/L, urea > 7 mmol/L), hepatic cirrhosis, acute or chronic hepatitis B or C, HIV, and chronic immunosuppressant therapy. In addition, patients will be excluded if they have received chemotherapy within four weeks of initiating immunogene therapy. Patients with a predicted survival of less than 3 months (as determined by their oncologist) or a Karnofsky performance scale of < 60 will be excluded.

3.3 c. Off-study criteria

A patient will be considered off treatment once protocol therapy has been stopped. Reasons for discontinuation of treatment include: development of NCIC CTG grade 3 or greater toxicity, patient has completed therapy, patient refuses further therapy, or patient develops other medical problems unrelated to the study that preclude further therapy.

The patient will be considered off-study in the event of death or initiation of non-protocol treatment. The patient who is off-study will be off treatment. However, a patient who is off treatment is not necessarily off-study. Patients who are off treatment but not off-study will continue to be followed for disease progression/response, late reports of toxicity and survival. Once a patient is off-study because of initiation of non-protocol treatment, the patient will be followed for survival purposes.

3.4 REGISTRATION AND INFORMED CONSENT

All patients will be enrolled in the study through the Walter C. Mackenzie Health Sciences Center prior to starting therapy. The following patient information is required: physician's name and phone number, institution's name, patient's name and hospital unit number, patient's date of birth, sex, race, height and weight, previous treatment (surgery, radiation therapy, chemotherapy) with dates, and the date and results of the pretreatment evaluations outlined above. The study coordinator (Dr. I. F. Parney) will review the eligibility checklist to verify patient eligibility. If the patient meets all criteria and consents to the study, they will be registered.

All patients must be aware of the neoplastic nature of his/her disease and willingly consent after being informed of the procedures to be performed, the experimental nature of the therapy, alternative therapies, potential benefits, side effects, risks, and discomforts. University of Alberta Research Ethics Board (REB) and federal Health Protection Branch (HPB) approval of this protocol and consent form is required.

3.5 Data collection and reporting of results

3.5 a. Adverse reactions to treatment

All symptoms and signs potentially secondary to therapy occuring while a patient is on study will be recorded and classified according to the NCIC NTG Expanded Common Toxicity Criteria. The following adverse reactions must be reported to the study chair, the institution's REB (Research Ethics Board), and the federal Health Protection Branch (HPB): all life-threatening and lethal (i.e.grade 4 and 5 toxicity) - telephone report within 24 hours and written report within 10 days; grade 2 and grade 3 toxicity - written report within 10 days. Written report, original NCIC NTG adverse drug reaction form, and three copies must be submitted to the study investigator within 5 days. Upon receipt the principal investigator will forward the original report to the HPB and copy to the REB. The study coordinator (Dr. I.F.Parney) and the research nurse (Ms. H. Vandenhoven) will be responsible for assessing and documenting any toxicities.

3.5 b. Immune responses to therapy

Local responses will be assessed through signs and symptoms of delayed-type hypersensitivity reactions at the vaccination sites and pathologic/ immunohistochemical examination of skin biopsies of the vaccination sites. The biopsies will be performed by the study coordinator, Dr. I.F. Parney. General inflammatory reactions will be monitored through serum C-reactive protein levels. Specific antitumor systemic responses will be assessed by assaying peripheral blood lymphocytes isolated from blood samples. These analyses will be performed in the principle investigators' laboratories at the University of Alberta.

3.5 c. Clinical response and quality of life assessment

History and physical examinations will be performed and compared with the pretreatment examination. Appropriate diagnostic imaging studies (i.e.

MRI with and without gadolinium enhancement) will be performed and compared to pretreatment studies. FACT version 3.0 scores will be recorded and compared with the pretreatment score. Medications and their doses (including steroids) and laboratory data will be recorded in accordance with the study parameters as stated. All of these assessments will be arranged by the study coordinator (Dr. I.F.Parney) and the research nurse (Ms. H. Vandenhoven) in consultation with the principle investigators and the patients' physicians.

3.5 d. Data collection and submission

The study coordinator (Dr. I.F. Parney) with the aid of the research nurse (Ms. H. Vandenhoven) will enroll patients in the study, give the vaccinations, take the skin biopsies, follow the study patients in the clinic, collect the relevant follow up data, and submit it to the principal investigator. Dr. Parney is a resident in the Division of Neurosurgery, a Ph.D. student in the Division of Experimental Surgery, and a Clinical Fellow of the Alberta Heritage Foundation for Medical Research.

Study forms will be submitted to the study principal investigator according to the following schedule:

On-study form	Within 1 week of registration
Treatment form	Within 1 week of treatment
Follow-up form	Every month while on study
Adverse reaction form	Within 5 days of evidence of toxicity

4.0 SUMMARY OF CLINICAL PROTOCOL

This clinical protocol is a pilot study of active tumor immunotherapy with irradiated autologous tumor cells modified to express the cytokine GM-CSF and the T cell costimulator molecule B7-2 (CD-86) in five patients with recurrent gliomas and five patients with melanoma resistant to conventional therapy. Our primary objective is to obtain information concerning the safety of such treatment. Secondary objectives include assessing immunologic responses to therapy and following clinical outcome (including quality of life). Toxicity and clinical outcomes are categorical (i.e. such as portions developing toxicity or obtaining complete or partial remissions) or measurements of duration (i.e. time to relapse or total survival). Immunologic outcomes are quantifiable (e.g. percent cytotoxicity, percentage of anti-tumor memory T cells). Information obtained from this study will assist in the ongoing design of phase II-III protocols where optimum biological doses and efficacy profiles will be determined. Furthermore, this study may provide a framework for subsequent gene modification studies directed at enhancing host systemic immune responses against refractory cancers.

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stimulating factor-transduced tumor cells, Human Gene Therapy. *8:* 1065-72, 1997.

Appendix 2

Curriculum Vitae

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Senior Resident, Division of Neurosurgery
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Personal Information

Date of Birth: May 13, 1969

Place of Birth: Calgary, Alberta, Canada
Citizenship: Canadian
Marital Status: Married

Neurosurgical Training

1993 - 1999

- Resident, Division of Neurosurgery, University of Alberta
- Edmonton, Alberta, Canada
- Program Director: Dr. J. M. Findlay
- Expected Completion Date: June 2001

Graduate Education

1995 - 1999

- Ph.D. Student, Division of Experimental Surgery, University of Alberta
- Edmonton, Alberta, Canada
- Supervisor: Dr. K.C. Petruk (Division of Neurosurgery)
- Thesis successfully defended October, 1998
- Expected Graduation Date: June 1999

Medical Education

1989 - 1993

- Faculty of Medicine, University of Alberta
- Edmonton, Alberta, Canada
- M.D. with Distinction granted June 1993
- B.Sc. with Distinction granted November 1991

Undergraduate Education

1986 - 1989

- Faculty of Science, McGill University
- Montreal, Quebec, Canada
- Major: Physiology
- Minor: Biomathematics

Honors

- 1997: K.G. McKenzie Prize for Basic Neuroscience Research, Canadian Neurosurgical Society
- 1997: Canadian Society for Clinical Investigation / Medical Research Council of Canada Residents' Research Award
- 1997: George R. Graham Bursary in Surgery, Department of Surgery, University of Alberta
- 1997: University of Alberta Hospitals Medical Resident Publication Prize
- 1997: Alpha Omega Alpha Medical Honor Society
- 1996 - 1998: Clinical Fellow, Alberta Heritage Foundation for Medical Research
- 1994: E.G. King Prize in Critical Care, Grey Nuns Hospital, Edmonton
- 1993: Graduating Class Ranking: First out of 126; Faculty of Medicine, University of Alberta
- 1993: Hewlett-Packard Top Medical Graduate Award, Faculty of Medicine, University of Alberta
- 1993: Boehringer Ingelheim Medical Achievement Award, Faculty of Medicine, University of Alberta
- 1993: A.E. Bowie Scholarship in Medicine for Otolaryngology, Faculty of Medicine, University of Alberta
- 1993: Sandoz Prize in Medicine for Neurology, Faculty of Medicine, University of Alberta
- 1989 - 1993: First Class Standing, Faculty of Medicine, University of Alberta
- 1990: Alberta Heritage Foundation for Medical Research Summer Studentship (Supervisor: Dr. T.G. Wegmann, Department of Immunology, University of Alberta)
- 1987: McConnell Award for Academic Excellence, McGill University
- 1986 - 1987: Faculty Scholar, Faculty of Science, McGill University

Publications

Characterization of Genetically Modified Human Brain Tumor Cell Cultures for Immunogene Therapy. I.F. Parney, A. Koshal, M. Cahill, M.A. Farr-Jones, L.-J. Chang, K.C. Petruk. In preparation, 1999.

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GM-CSF and B7-2 Combination Immunogene Therapy in an Allogeneic Hu-PBL-SCID Mouse - Human Glioblastoma Multiforme Model. I.F. Parney, K.C. Petruk, C.S. Zhang, D. Sykes, M.A. Farr-Jones, L.-J. Chang. **Human Gene Therapy** 8: 1073-1085, 1997.

"Idiopathic" Cranial Hypertrophic Pachymeningitis Responsive to Antituberculous Medications: Case Report. I.F. Parney, E.S. Johnson, P.B.R. Allen. **Neurosurgery** 41: 965-971, 1997.

Abstracts

B7-2 and GM-CSF but not IL12-Transduced Human Glioma Cells Synergize with Wild Type to Stimulate Autologous Antitumor Lymphocytes In Vitro. I.F. Parney, K. Kane, D.-E. Gong, M.A. Farr-Jones, L.-J. Chang, K.C. Petruk. Congress of Neurological Surgeons, Seattle, Washington, October, 1998.

Characterization of Genetically Modified Human Brain Tumor Cell Cultures for Immunogene Therapy. I.F. Parney, A. Koshal, M. Cahill, M.A. Farr-Jones, L.-J. Chang, K.C. Petruk. Congress of Neurological Surgeons, Seattle, Washington, October, 1998.

Dr. H.H. Hepburn and the Development of Cervical Spine Traction at the University of Alberta. I.F. Parney, P.B.R. Allen, K.C. Petruk. Canadian Congress of Neurologic Sciences, Montreal, Quebec, June 1998.

Human Glioma Fas / Fas-Ligand Expression. I.F. Parney, J.F. Elliott, M.A. Farr-Jones, K.C. Petruk. American Association of Neurological Surgeons, Philadelphia, Pennsylvania, April 1998.

Combination Immunogene Therapy for Gliomas. I.F. Parney, K.C. Petruk, M.A. Farr-Jones, C.S. Zhang, L.-J. Chang. Twelfth International Conference on Brain Tumour Research and Therapy, Oxford, U.K., September 1997.

K.G.McKenzie Lecture: Combination Immunogene Therapy for Glioblastoma Multiforme. I.F. Parney, K.C. Petruk, M.A. Farr-Jones, C.S. Zhang, L.-J. Chang. Canadian Congress of Neurological Sciences. Saskatoon, Saskatchewan, June 1997.

GM-CSF and B7-2 Combination Immunogene Therapy in an Allogeneic Hu-PBL-SCID Mouse - Human Glioblastoma Multiforme Model. I.F. Parney, K.C. Petruk, M.A. Farr-Jones, C.S. Zhang, L.-J. Chang. American Association of Neurological Surgeons. Denver, Colorado, April 1997.

Immunobiology of Human Glioblastoma Multiforme: Implications for Gene Therapy. I.F. Parney, M.A. Farr-Jones, L.-J.Chang, K.C. Petruk. American Association of Neurological Surgeons. Denver, Colorado, April 1997.

Improved Processing Technique for Establishing Human Glioblastoma Multiforme Explant Cultures. I.F. Parney, M.A. Farr-Jones, K.C. Petruk. American Association of Neurological Surgeons. Denver, Colorado, April 1997.

Combination Immunogene Therapy Using Multicistronic Vectors Carrying T-Cell Costimulator B7-2, GM-CS, and IL-12 for Gene Transfer to Heterologous Human Tumors. L.-J. Chang, I.F. Parney, Y. Cui, J. Chua, K.C. Petruk. Second Annual Conference on Gene Therapy. Cold Spring Harbor, October 1996.

Preliminary Models of Immunogene Therapy for Glioblastoma Multiforme. I.F. Parney, K.C. Petruk, M.A. Farr-Jones, L.-J. Chang. Canadian Congress of Neurological Sciences. London, Ontario, June 1996.

Teaching Experience

- March 22, 1999: Lecturer, Department of Respiratory Therapy, Northern Alberta Institute of Technology; "Head Injuries"
- December 14, 1998: Lecturer, Division of Pediatric Nursing, University of Alberta Hospital; "Pediatric Head Injuries"
- April 3, 1998; Lecturer, Faculty of Medicine (Neuroscience 410), University of Alberta; "Hemorrhagic Cerebrovascular Disease"
- November 18, 1997: Lecturer, Division of Radiation Oncology, University of Alberta; "Surgical Aspects of Central Nervous System Neoplasms"
- Summer student co-supervisor:
    - April - September 1998: Mr. Anu Koshal, Ms. Annie Young, Mr. C. O' Kelly
    - April - September 1997: Ms. Colleen Hope, Mr. Anu Koshal
- Undergraduate "Honors in Research" student co-supervisor:
    - September - December 1997: Mr. Mark Cahill

Professional Associations

- Canadian Society for Clinical Investigation, 1997 - present
- AANS / CNS Joint Section on Tumors, 1997 - present
- Congress of Neurologic Surgeons, 1996 - present
- American Association of Neurological Surgeons, 1996 - present
- Canadian Medical Association, 1989 - present
- Alberta Medical Association, 1989 - present

Additional Certifications

- Licentiate of the Medical Council of Canada (granted 1994)
- Advanced Trauma Life Support
- Advanced Neonatal Life Support
- Advanced Cardiac Life Support

References

Excellent references available upon request.